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# THE INFLUENCE OF PREGNANCY UPON THE LIPOIDS OF THE BLOOD.

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(Received for publication, May 23, 1925.)

The data herein presented furnish, we believe, reliable information relative to alterations in blood lipoids during pregnancy and the first 2 weeks of the puerperium. Beginning as early as the 3rd month the total ether extract of whole blood of pregnant women is higher than that of non-pregnant women, the increase being progressively augmented until term. Cholesterol, cholesterol esters, and lecithin increase gradually until term, when each is approximately one-third higher than at the 3rd month of pregnancy. During the period of pregnancy, the relation of cholesterol esters to the total cholesterol remains fairly constant, and the esters form a larger percentage of the total cholesterol than obtains in non-pregnant women. The relation of lecithin to cholesterol remains fairly constant during pregnancy, and is at all times within the normal limits for that value in non-pregnant women. During the first 2 weeks of the puerperium the values for the ether extract and for the individual lipoids of whole blood remain high.

In the literature are certain references which, in as far as they are applicable to our own experiments, are in harmony with the conclusions stated above. Thus, in 1904, Capaldi, in an Italian journal, noted that the blood fat of dogs late in pregnancy was almost double that of the non-pregnant animal. This is quoted by Bar, in 1907, who adds his own observation that serum of pregnant women appears white, and as there is an abundance of fat in the viscera at this time, he is not surprised to find it increased in the blood.

Herrmann and Neumann (1912) give figures to prove their statement that cholesterol and neutral fat are increased at term; the "cholesterol fat" increasing 68.6 per cent and the "glycerol fat," 80 per cent. The outstanding earlier worker in this field is Chauffard (13), who published with various collaborators a series of papers on cholesterol in the blood of normal persons, in various diseases, and in pregnancy. He analyzed the serum of pregnant women in various months of gestation; thirteen of these are under 7 months. His conclusions are that during the first 6 to 7 months the serum cholesterol may be increased, and that during the last 2 months (thirty-two cases) an increase in serum cholesterol is the rule.

Denis (14) gives cholesterol values for a number of early pregnancies—the exact month is not given. These figures are interpreted as normal, although they seem to us rather above the normal.

Bloor (8) mentions the fact that total fat and cholesterol are increased in pregnancy and gives cholesterol values for three cases probably at term. Slemons (22) has published blood fat, cholesterol, and lecithin determinations of a large series of women at the conclusion of labor; these show a marked increase in all lipoids over the non-pregnant findings. He also reports a few determinations earlier in pregnancy; these are in cases of pernicious vomiting, threatened abortion, or other abnormality. Plass and Tompkins (18) also have given figures for blood and lipoids, particularly cholesterol, during pregnancy. These figures indicate a gradual rise from the 4th month to term.

Of special interest to us are the results of cholesterol estimations in conditions associated with growth, whether in the form of tumors or in physiological growth. Bloor has found cholesterol increased in the blood corpuscles of persons with cancer (6); V. C. Myers also reports high cholesterol in the blood of persons with new growths; Robertson injected cholesterol directly into tumors in white rats and found a considerable increase in growth resulting therefrom (21). He also fed mice cholesterol, and reports it to be at times a stimulus to growth (20). Cholesterol furthermore is said to cause marked increase in the rate of division of the paramecium (11). In all these instances, together with the reported increase in pregnancy, we have conditions of cell multi-

plication, whether pathological as cancer, or physiological as pregnancy and growth, associated with high cholesterol.

## EXPERIMENTAL OBSERVATIONS.

### *Methods.*

For our work ten women were chosen from each lunar month of pregnancy. The better method of choosing a certain number of women very early in pregnancy and obtaining a sample of blood month by month was abandoned, as it was too rigorous for our dispensary patients. A few of our women have served two

TABLE I.

*Blood Lipoids in Non-Pregnant Women.*

Values expressed in mg. per 100 gm. of whole blood.

| Case No. | Total ether extract. | Total cholesterol. | Cholesterol esters. | Lecithin. |
|---|---|---|---|---|
| | *mg. per 100 gm.* | *mg. per 100 gm.* | *mg. per 100 gm.* | *mg. per 100 gm.* |
| 93 | 622 | 216 | 87 | |
| 94 | 608 | 188 | 65 | 250 |
| 95 | 623 | 183 | 76 | 304 |
| 96 | 647 | 231 | 106 | 299 |
| 97 | 582 | 212 | 80 | 271 |
| 98 | 546 | 190 | 82 | 320 |
| 99 | 575 | 232 | | 370 |
| 100 | 606 | 180 | 81 | 280 |
| 101 | 734 | 188 | 75 | 333 |
| 102 | 747 | 249 | | 320 |
| Average.... | 629 | 207 | 81 | 305 |

or more times, but the majority only once. Great care was taken to have them come to the dispensary without breakfast, allowing them, however, a cup of clear coffee. The blood for lipoid determination was then taken from the arm vein at the same time as the routine Wassermann test. Samples were taken in like manner from hospital patients during the first 2 weeks postpartum—ten women on the 2nd day following delivery, and from ten women near the end of the 2nd week, when the milk supply had become thoroughly established. No women are included in our series who had positive Wassermann reactions or any pathological condition, either in the general physical

TABLE II.

*Blood Lipoids during Pregnancy.*

Values expressed in mg. per 100 gm. of whole blood.

| Case No. | Total ether extract. | Total cholesterol. | Cholesterol esters. | Lecithin. |
|---|---|---|---|---|
| | | 3 months. | | |
| | *mg. per 100 gm.* | *mg. per 100 gm.* | *mg. per 100 gm.* | *mg. per 100 gm.* |
| 1 | | 209 | 83 | 300 |
| 2 | 588 | 153 | 82 | 438 |
| 3 | 660 | 156 | 79 | 298 |
| 4 | 780 | 214 | 85 | 240 |
| 5 | 890 | 203 | 92.8 | 270 |
| 6 | 809 | 201 | 60 | 233 |
| 7 | 856 | 210 | 78 | 295 |
| 8 | 815 | 187 | 93.7 | 267 |
| 9 | 512 | 188 | 102 | 252 |
| 10 | 741 | 192 | 100 | 258 |
| Average.... | 739 | 191 | 86 | 285 |
| | | 4 months. | | |
| 11 | | 190 | 115 | 428 |
| 12 | 810 | 213 | 126 | 318 |
| 13 | 925 | 276 | 141 | 391 |
| 14 | 748 | 174 | 92 | 230 |
| 15 | 515 | 178 | 111 | 245 |
| 16 | 673 | 229 | 85 | |
| 17 | 685 | 173 | 56 | |
| 18 | 753 | 229 | 95 | 369 |
| 19 | 572 | 206 | 106 | |
| Average.... | 710 | 207 | 103 | 330 |
| | | 5 months. | | |
| 20 | 882 | 168 | 81 | 390 |
| 21 | 674 | 196 | 100 | 273 |
| 22 | 700 | 161 | 70 | 270 |
| 23 | 768 | 224 | 121 | 356 |
| 24 | 780 | 240 | 106 | 387 |
| 25 | 625 | 230 | 95 | 361 |
| 26 | 933 | 174 | 106 | 300 |
| 27 | 740 | 243 | 138 | 300 |
| 28 | 794 | 224 | 100 | |
| 29 | 832 | 215 | 132 | 359 |
| Average.... | 773 | 207 | 105 | 333 |

TABLE II—*Continued.*

| Case No. | Total ether extract. | Total cholesterol. | Cholesterol esters. | Lecithin. |
|---|---|---|---|---|
| | | 6 months. | | |
| | *mg. per 100 gm.* | *mg. per 100 gm.* | *mg. per 100 gm.* | *mg. per 100 gm.* |
| 30 | 791 | 260 | 113 | 273 |
| 11 | 870 | 268 | 117 | 342 |
| 31 | 930 | 210 | 96 | |
| 32 | 670 | 140 | 88 | |
| 33 | 925 | 209 | 95 | 331 |
| 34 | 721 | 228 | 138 | 290 |
| 35 | 705 | 190 | 80 | 260 |
| 36 | 918 | 192 | 90 | 300 |
| 37 | 743 | 196 | 148 | 265 |
| 38 | 804 | 202 | 95 | 341 |
| Average.... | 808 | 210 | 106 | 300 |
| | | 7 months. | | |
| 39 | 910 | 225 | 111 | 443 |
| 40 | 975 | 178 | 70 | 353 |
| 11 | 800 | 248 | 142 | 337 |
| 41 | 707 | 298 | | 378 |
| 42 | 810 | 240 | 140 | 330 |
| 43 | 708 | 234 | 108 | 342 |
| 44 | 814 | 272 | 156 | 336 |
| 16 | 1006 | 294 | 130 | 400 |
| 45 | 771 | 201 | 98 | 283 |
| 46 | 544 | 226 | 134 | 337 |
| Average.... | 804 | 242 | 109 | 354 |
| | | 8 months. | | |
| 47 | 1016 | 195 | 68 | |
| 48 | 1010 | 232 | 93 | 424 |
| 49 | 693 | 250 | 113 | 285 |
| 50 | 750 | 217 | 140 | |
| 51 | 868 | 194 | 88 | 454 |
| 52 | 725 | 238 | 97 | 375 |
| 53 | 1088 | 245 | 100 | 456 |
| 54 | 769 | 200 | 87 | 318 |
| 11 | 800 | 264 | 108 | 430 |
| 55 | 784 | 338 | 167 | 286 |
| 56 | 693 | 220 | 100 | 276 |
| 57 | 725 | 171 | 132 | 275 |
| Average.... | 827 | 230 | 108 | 358 |

TABLE II—*Concluded.*

| Case No. | Total ether extract. | Total cholesterol. | Cholesterol esters. | Lecithin. |
|---|---|---|---|---|
| | | 9 months. | | |
| | *mg. per 100 gm.* | *mg. per 100 gm.* | *mg. per 100 gm.* | *mg. per 100 gm.* |
| 58 | 900 | 227 | 69 | |
| 59 | 970 | 270 | 118 | 325 |
| 60 | 998 | 304 | 76 | 366 |
| 61 | 780 | 241 | 134 | 300 |
| 62 | 700 | 210 | 94 | |
| 63 | 700 | 220 | 114 | 350 |
| 64 | 861 | 239 | 129 | 400 |
| 65 | 732 | 231 | 114 | 305 |
| 66 | 743 | | 118 | 213 |
| 67 | | 257 | 115 | 311 |
| Average.... | 820 | 244 | 108 | 321 |
| | | Term. | | |
| 68 | 1055 | 300 | 149 | |
| 69 | 824 | 318 | 160 | 460 |
| 70 | 1145 | 236 | 122 | 400 |
| 71 | 909 | 195 | 87 | 341 |
| 72 | 807 | 222 | 125 | 331 |
| 73 | 767 | 300 | | |
| 74 | 888 | 236 | 142 | 272 |
| 75 | 884 | 222 | 138 | 335 |
| 76 | 881 | 332 | 150 | 389 |
| 77 | 1087 | 240 | 136 | 262 |
| 45 | | 316 | 222 | |
| 38 | 982 | 213 | 90 | |
| Average.... | 930 | 261 | 138 | 361 |

examination or in the pregnancy. The bloods of ten normal non-pregnant women in the child-bearing age were used as controls. These samples were taken before breakfast was eaten and midway between menstrual periods, in order to eliminate any possible lipoid variation which has been reported to occur during menstruation (17).

On each sample were determined the total ether extract, total cholesterol, cholesterol esters, and lecithin. We followed Bloor's directions for determination of the total ether extract (3), total

cholesterol (5), and cholesterol esters (9). For lipoid phosphoric acid or lecithin, we followed Bloor's method early in the work (7), but later this was replaced by the method of Randles and Knudson (19), which was shorter and simpler for execution, and gave results which checked with the earlier method. Whole blood was used in all our work. All determinations were done in duplicate or until results checked; two samples of blood were used from each patient.

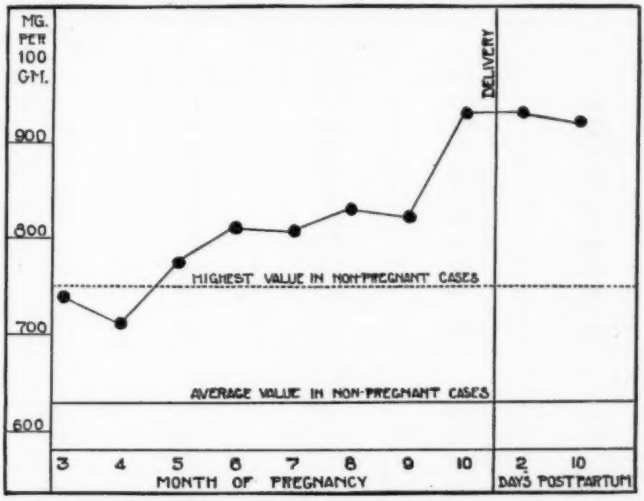


FIG. 1. Total ether extract of whole blood. Each dot represents the average value for total ether extract in whole blood of women in the various months of pregnancy and during the puerperium. For comparison the average value and the highest single value obtained in non-pregnant women are shown by the two horizontal lines.

## DISCUSSION.

### *Total Ether Extract.*

The values for non-pregnant individuals vary between 546 and 747 mg. per 100 gm. of whole blood, with an average value of 629 (Table I). Values of the total ether extract for the 3rd month of pregnancy already show both an increase and a wider individual variation—the figures varying between 512 and 890,

with an average of 739 (Table II and Fig. 1). The next 2 months give essentially the same breadth of excursion and average values. From the 6th month till the end of pregnancy the average values become higher, also the highest values of each month are higher,

TABLE III.

*Blood Lipoids during the Puerperium.*

Values expressed in mg. per 100 gm. of whole blood.

| Case No. | Total ether extract. | Total cholesterol. | Cholesterol esters. | Lecithin. |
|---|---|---|---|---|
| | 2 days postpartum. | | | |
| | *mg. per 100 gm.* | *mg. per 100 gm.* | *mg. per 100 gm.* | *mg. per 100 gm.* |
| 78 | 992 | 243 | 90 | |
| 79 | 964 | 319 | 154 | 350 |
| 80 | 890 | 235 | 116 | 497 |
| 81 | 1090 | 221 | 93 | |
| 82 | 1010 | 236 | 110 | 468 |
| 83 | 888 | 218 | 87 | 325 |
| 84 | 737 | 257 | 139 | 350 |
| 85 | 932 | 277 | 168 | 265 |
| 86 | 866 | 275 | 140 | 304 |
| Average.... | 930 | 253 | 122 | 366 |
| | 10 to 12 days postpartum. | | | |
| 87 | 1300 | 274 | 114 | 390 |
| 88 | 992 | 241 | 91 | 384 |
| 89 | 1100 | 319 | 118 | 360 |
| 90 | 1230 | 270 | 185 | 325 |
| 91 | 820 | 246 | 124 | 367 |
| 63 | 854 | 242 | 127 | 318 |
| 92 | 945 | 290 | 120 | 452 |
| 11 | 590 | 230 | 96 | 393 |
| 41 | 762 | 277 | 129 | 380 |
| 23 | 845 | 305 | 172 | 449 |
| 25 | 672 | 385 | 153 | 280 |
| Average.... | 919 | 280 | 130 | 373 |

and the lowest values for each month are higher than in the earlier months. Out of fifty estimations during the last 4 months of pregnancy, there is only one value below 629, which is the average for non-pregnant women. The highest values during

pregnancy occur in women at term, at which time the average of ten cases was 930, the highest 1145, and the lowest 767. During the 2 weeks following delivery, the values remain high (Table III). Beyond this date we have not followed our patients.

*Total Cholesterol.*

Our figures for cholesterol are roughly parallel to our figures for the total ether extract (Fig. 2 and Tables II and III). The

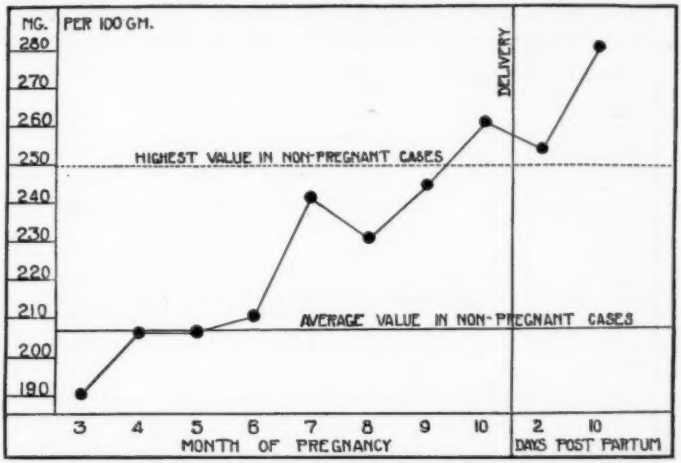


FIG. 2. Total cholesterol in whole blood. The dots represent the average value for total cholesterol in the blood of women at various months of pregnancy and in the puerperium.

average value for total cholesterol in the non-pregnant women is 207 mg. per 100 gm. of blood, while from the 3rd to the 6th month our averages are 191, 207, 207, and 210 for the successive months. From then on there is a marked increase, the highest figure being reached 10 days postpartum, instead of at term, as was the case with the total ether extract. The increase in cholesterol from early pregnancy, or the non-pregnant condition to 10 days postpartum, is over one-third, practically the same proportion as with the total ether extract. As was true with the latter, in that period of pregnancy when the average value for

cholesterol is the greatest, that average is above the highest single value for cholesterol in the blood of any non-pregnant woman.

*Cholesterol Esters.*

These values also show a rise as pregnancy progresses, attaining the greatest height at term, and remaining high during the following 2 weeks. Here the final gain is over 50 per cent of the value in the non-pregnant cases (Fig. 3).

The relation between total cholesterol and cholesterol bound as esters should remain the same if both increase during preg-

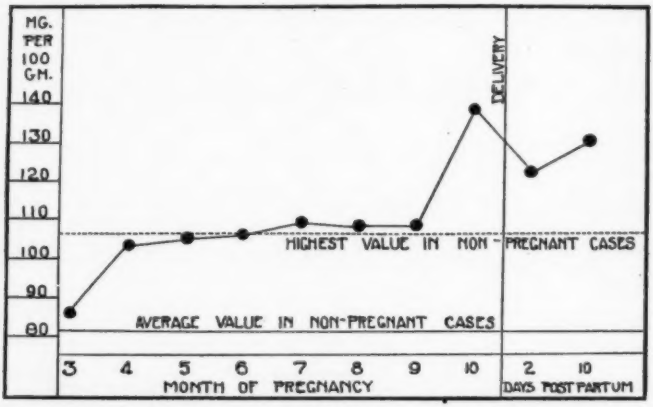


FIG. 3. Cholesterol esters in whole blood. Each dot represents the average value for cholesterol esters in the blood of women at various months of pregnancy and during the puerperium.

nancy at the same rate. This is found roughly to be the case; that is, for the different months cholesterol as esters varies between 44 and 53 per cent of the total cholesterol in the blood. This is a higher percentage than that found in non-pregnant women in whom the average percentage of cholesterol as esters was 39 (Table IV). Bloor and Knudson (10) give the percentage of cholesterol as esters in whole blood as 33.8 for men and 33.3 for women, with variations for the latter from 30 to 42. In pathological conditions they find the relationship generally remaining constant. Even in diabetes, where values for choles-

terol are four or five times the normal, the relationship remains the same. In three cases of pregnancy, however, the cholesterol esters are reported high, representing 54.9, 47, and 60 per cent of the total cholesterol.

TABLE IV.

| Condition of patient. | Total cholesterol as esters.* | Ratio: $\frac{\text{Lecithin}}{\text{Cholesterol}}$* |
|---|---|---|
| | *per cent* | |
| Pregnant 3 mos. | 45 | 1.49 |
| " 4 " | 49.7 | 1.59 |
| " 5 " | 50.7 | 1.61 |
| " 6 " | 50.5 | 1.43 |
| " 7 " | 45 | 1.46 |
| " 8 " | 47 | 1.55 |
| " 9 " | 44.3 | 1.32 |
| " 10 " | 53 | 1.38 |
| Postpartum 2 days. | 48.2 | 1.44 |
| " 10 " | 46.4 | 1.33 |
| Non-pregnant. | 39.1 | 1.47 |

* Each number is the average of all the cases in that particular group or period.

TABLE V.

*Lipoids of Whole Blood.*

| | Total ether extract. | | | Cholesterol. | | Lecithin. | |
|---|---|---|---|---|---|---|---|
| | High. | Low. | Average. | High. | Low. | High. | Low. |
| 24 cases at conclusion of labor (Slemons and Stander)..... | 1120 | 740 | 883 | 330 | 190 | 350 | 265 |
| 12 women at term (authors) | 1145 | 767 | 930 | 332 | 195 | 460 | 262 |

*Lecithin.*

Our average for lecithin in non-pregnant women is 305 mg. per 100 gm. of whole blood and our highest group average, 10 days postpartum, 373 mg. per 100 gm. of blood. Lecithin shows an increase, as do the other blood lipoids, although more gradual and with irregularities that might be obliterated in a larger series of cases (Fig. 4). The gain in lecithin from the value at 3 months to that at term, is about one-third. In normal indi-

viduals there is a constant relation between the lecithin and cholesterol of the blood; according to Bloor (8) the value $\frac{\text{lecithin}}{\text{cholesterol}}$ being 1.44 in whole blood for men, and 1.29 for women, with individuals varying from 1.63 to 1.20 (men) and 1.33 to 1.21 (women). He further states that in persistent lipemia—notably diabetes, when both lecithin and cholesterol are increased—this ratio is usually low, due to the greater increase of cholesterol over lecithin. The averages of our cases at the various months of pregnancy give figures which are a little higher than our normal of 1.47 in early pregnancy, and lower late in pregnancy

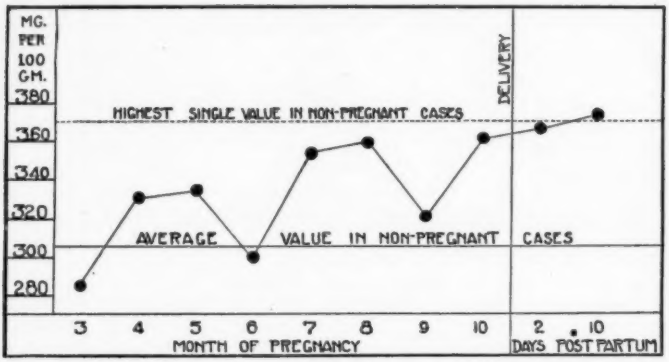


FIG. 4. Lecithin in whole blood. Each dot represents the average value for lecithin in the blood of women at various months of pregnancy and in the puerperium.

(Table IV). The difference is very slight, exceeded by the variations among our individual non-pregnant cases, so it is probably of no moment. In other words, our series of pregnant women shows a gain in lecithin that progresses at about the same rate as the increase in cholesterol.

The total ether extract of whole blood increases, then, steadily and more or less regularly during pregnancy. The individual lipoids, total cholesterol, cholesterol esters, and lecithin increase at approximately the same rate. The individual variation in blood lipoids of non-pregnant women is accentuated during pregnancy, the range in any 1 month being greater than in women

who are not pregnant. In Table V are given the figures from Slemons and Stander of patients at the end of labor. They represent twenty-four cases, some of these spontaneous deliveries, others operative, some with anesthetic, others without; their results are expressed in mg. per 100 cc. of blood, while ours are in mg. per 100 gm. of blood, which would necessitate roughly a 5 per cent correction. The similarity between our results of twelve cases at term and theirs is striking.

## CONCLUSIONS.

1. The total ether extract of whole blood in pregnant women is higher than the non-pregnant values as early as the 3rd month of pregnancy and there is an increase from then till term. The average value for the total ether extract in eleven cases at term is roughly 50 per cent higher than the non-pregnant values and 30 per cent higher than the value at 3 months.

2. Cholesterol, cholesterol esters, and lecithin increase gradually until term, when each is roughly one-third higher than at 3 months.

3. During pregnancy, the relation of cholesterol esters to the total cholesterol remains fairly constant, and the esters form a larger percentage of the total cholesterol than in non-pregnant women.

4. The relation $\frac{\text{lecithin}}{\text{cholesterol}}$ remains fairly constant during pregnancy, and is at all times within the normal limit for that value in non-pregnant women.

5. During the first 2 weeks of the puerperium the values for the ether extract and for the individual lipoids of whole blood remain high.

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# A NOTE ON THE TECHNIQUE FOR STUDYING VITAMIN B.

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(Received for publication, July 20, 1925.)

Although for more than a decade the vitamins have been recognized as dietary essentials and although numerous experimental data on the distribution of these food factors have been gathered, the satisfactory methods available for their detection and relative quantitative determination are singularly few. Various techniques, such as the yeast growth tests, have been devised in an attempt to obviate the use of animals, but they have not proven satisfactory. Today the most desirable and accurate means of testing for the vitamins involves the use of rats, guinea pigs, mice, or pigeons.

In 1923, Steenbock, Sell, and Nelson pointed out the fact, which seems clear from their data, that in tests for vitamin B carried out with rats, considerable variation is found in the quantity of vitamin-containing material required for normal growth, depending on the method of caging the rats. When the experimental animals had access to their excreta, demonstrably smaller quantities of the vitamin-containing materials were required to bring about resumption of growth in the curative tests than when the rats were prevented from eating their feces. This indicated either that part of the ingested food factor was excreted unchanged or that the bacteria in the intestine synthesized the vitamin in the course of their growth processes. Corroborative studies on the rat have been made recently by Dutcher and Francis (1923–24), and Cowgill, Deuel, and Smith (1925) have given such findings a wider significance by their report of similar phenomena shown by the dog.

This suggestion of improved technique made by the Wiscon-

sin investigators has been severely criticized by McCollum, Simmonds, and Becker (1925), who account for the difference in results partly by the failure to clean the old-style cage frequently enough and partly by the discomfort of the rats resting on the wire mesh false-bottom of the new-style cage.

In the course of many investigations carried out in this laboratory involving the use of the rat in vitamin tests, ample opportunity has been offered to compare the behavior of these animals in the two types of cages under otherwise identical, exceptionally well controlled conditions.

In view of the criticisms offered by McCollum, Simmonds, and Becker, it seems worth while to describe in some detail the technique employed in this laboratory. Each experimental animal is kept in an individual all metal cage. What we shall call our old-style cage consists of a cylinder of $\frac{1}{4}$ inch mesh wire cloth resting in an ordinary granite-ware pudding pan. On the bottom of the pan is a piece of absorbent paper covered with a piece of wire gauze of $\frac{1}{8}$ inch mesh which prevents the rat from eating the paper. Food and water are contained in glass cups set in galvanized iron rings attached to the side of the cage. The old-style cage is open at the bottom and the rat rests on the wire gauze in the bottom of the pan, with access to feces. This cage has been described in detail and photographs have been published by Ferry (1919–20). Following the suggestion of Steenbock, Sell, and Nelson that the feces contain vitamin B, a new cage was designed for use in studies of this accessory factor. In the new cages a false-bottom of $\frac{1}{4}$ inch mesh wire gauze is soldered into the cage proper, thus raising the rat a distance of 1 inch from the bottom of the pan and preventing access to feces. A removable cover with handle is provided for the top. Once each week every cage in the rat room is disassembled, each part washed and subjected to live steam for $\frac{1}{2}$ hour. At semiweekly periods the pan with its gauze is washed and steamed. Thus the parts of the cages most soiled by the rats are thoroughly cleaned twice each week. In both types of cage the rat rests on wire gauze of almost the same size mesh. Any difference in behavior of the rat due to discomfort is thus ruled out. The comparisons of the two types of cages are likewise not affected by any difference in frequency or care of cleaning. It is possible

that animals confined in false-bottom cages such as McCollum, Simmonds, and Becker describe with as large a mesh as ½ inch would suffer considerable discomfort. As stated above, the mesh of the false-bottom of our cages is ¼ inch.

Although none of the experiments here reported was designed

TABLE I.

*Quantity of Vitamine (Harris) Powder* Required per Day for Normal Growth to 120 Gm. Body Weight.*

| Old-style cage. | | False-bottom cage. | |
|---|---|---|---|
| Rat No. | Quantity. | Rat No. | Quantity. |
| | *mg.* | | *mg.* |
| 57 | 20 | 860 | 30 |
| 66 | 20 | 859 | 30 |
| 68 | 20 | 858 | 30 |
| 69 | 20 | 853 | 45 |
| | | 856 | 30 |
| | | 855 | 30 |
| Average..... | 20 | Average..... | 33 |

* Prepared by the Harris Laboratories, Tuckahoe, New York.

TABLE II.

*Quantity of Vitavose* Required per Day as a Source of Vitamin B for Normal Growth to 150 Gm. Body Weight.*

| Old-style cage. | | False-bottom cage. | |
|---|---|---|---|
| Rat No. | Quantity. | Rat No. | Quantity. |
| | *mg.* | | *mg.* |
| 900 | 200 | 896 | 400 |
| 901 | 300 | 897 | 300 |
| 902 | 200 | 898 | 300 |
| 903 | 200 | 899 | 400 |
| Average..... | 225 | Average..... | 350 |

* Hoffman, C., *Ind. and Eng. Chem.*, 1925, xvii, 498.

primarily to test the thesis in question, results obtained from feeding trials in which the old-style and false-bottom cages were compared, show very clearly that the feces may contain appreciable amounts of vitamin B. In Tables I and II is shown the difference in requirement for two products containing vitamin B which were the sole source of that food factor in the diet used, when the old-style cage and the false-bottom cage were employed.

It is obvious from the data that when the experimental animals were prevented from eating their feces, definitely increased

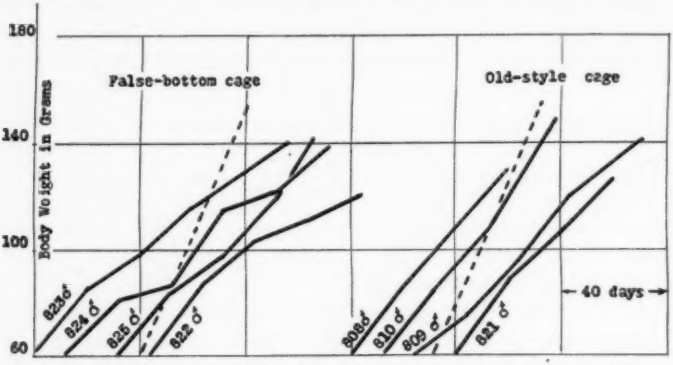


CHART I. Effect of method of caging on growth. Normal growth rate indicated by dotted line.

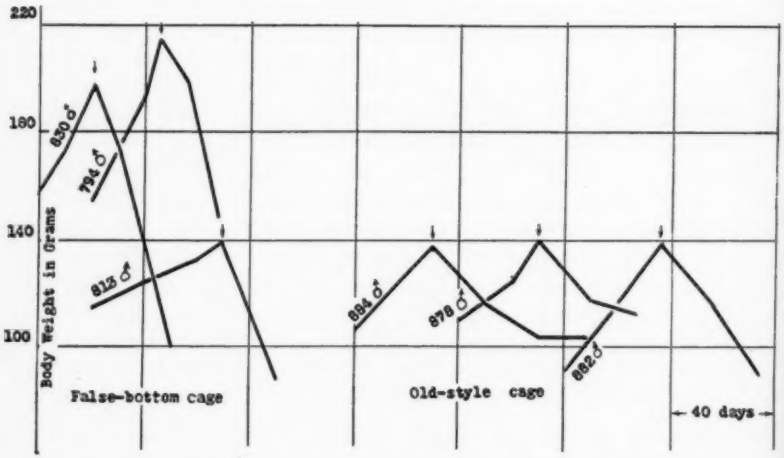


CHART II. Effect of method of caging on rate of decline on a diet with inadequate vitamin content. The vitamin-bearing material was removed from food at ↓.

daily dosages of the vitamin-bearing material were required for normal growth.

In Chart I are shown the growth curves of two series of rats, both eating a synthetic food in which 30 per cent of yellow whole corn was the only source of vitamin B, but one group of which had access to feces while the other was kept in false-bottom cages. The growth rate is distinctly superior with the animals in the old-style cages.

In Chart II is shown another type of evidence which may be used to compare the effect of the difference in system of caging. Both groups of rats were growing on identical adequate food mixtures and at the point indicated the source of vitamin B was removed. It is seen that the rate of decline is more rapid with the animals confined in the false-bottom cages.

Chart III illustrates the point somewhat differently. Two of the animals in the old-style cage were given 30 mg. of Vitamine Harris powder daily after they had declined in body weight on a diet lacking vitamin B; the other two were given the same dosage of the same material from the beginning of the experiment. Growth at the normal rate to approximately 175 gm. body weight was obtained in all the rats. The group of rats on the false-bottom cage was started on an otherwise adequate food plus 20 mg. of Vitamine Harris daily. This dosage was increased to 30 mg. when growth ceased. In no case did the body weight reach 150 gm. on this latter amount of the vitamin-containing substance—a definite and marked contrast to the behavior of the rats having access to feces.

These types of evidence have been repeatedly duplicated in our experience. We have noted a more speedy failure of very young rats on vitamin B-free diets when confined in false-bottom cages than when kept in the old-style cage with access to excreta. *Daily* removal of feces from a cage not otherwise constructed to prevent coprophagy is not frequent enough, for rats suffering from lack of vitamin B will seize and consume fecal pieces with astonishing quickness and dexterity. This has also been observed by Steenbock and his associates. On the basis of our experience, therefore, we are prepared to defend the idea that the animal-feeding method of testing for vitamin B can give accurate *absolute* results for a given product only when the experimental animal is prevented from conserving his supply of this food factor by ingesting feces. In this we confirm Steen-

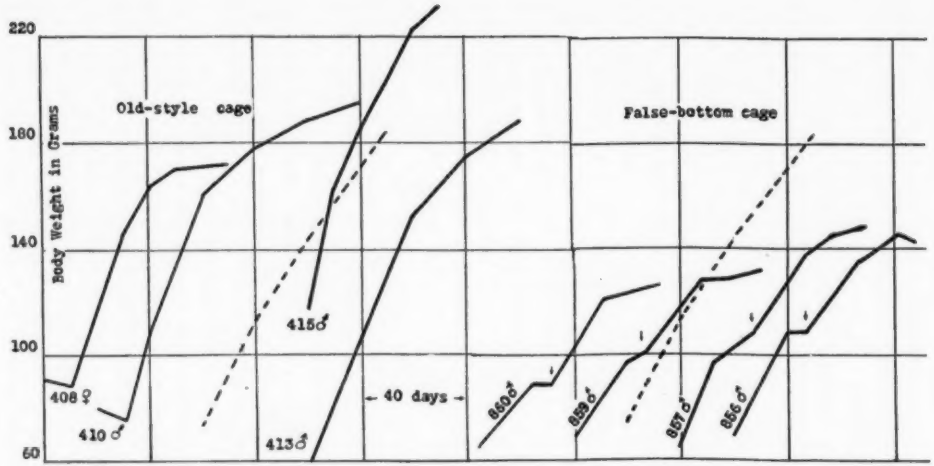


CHART III. Effect of method of caging on amount of vitamin-containing material required for definite gains in body weight. At ↓ the daily dose was increased to 30 mg. Normal growth rate indicated by dotted line. Animals in old-style cages had 30 mg. from beginning.

bock, Sell, and Nelson. This point will be all the more important when vitamin B is isolated in pure form.

On the other hand, the method of testing hitherto employed possesses value if only comparative results not highly quantitative in character are desired. When, however, one wishes to determine as accurately as possible the minimum amount of a given source of vitamin B necessary to satisfy the experimental animal's requirement, we believe that coprophagy must be guarded against. In addition we would emphasize the necessity of frequent, thorough cleaning. This is even more important when the method of experimentation employed allows several animals to be caged together. From the standpoint of sanitation as well as of clear-cut experimental results, it is highly desirable to cage each animal separately. The advantages of this procedure include not only a cleaner cage, but also opportunity to measure with some degree of accuracy the amount of food eaten by each animal; and the feeding experiments are given a quantitative character as well as a merely qualitative one. We believe that this procedure rather than the group-caging method is amply justified by the accumulating evidence pointing to the fact that the rat is as satisfactory for intensive experimentation under the right conditions as are some of the larger laboratory animals.

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# THE SOLUBILITY OF TERTIARY CALCIUM PHOSPHATE IN CEREBROSPINAL FLUID.

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(Received for publication, July 28, 1925.)

It was shown in a recent publication (1) that blood serum is normally supersaturated with tertiary calcium phosphate, and the suggestion was made that other body fluids are similarly supersaturated with this salt. In order to confirm this idea, solubility experiments were undertaken with cerebrospinal fluid; it was furthermore hoped that these studies might throw some light on the question of the distribution of calcium and phosphorus between blood serum and spinal fluid.

## EXPERIMENTAL.

### *General Procedure.*

Specimens of pooled human spinal fluid were shaken continuously for 7 to 9 days at 38°C. in the presence of solid $Ca_3(PO_4)_2$. The fluid was covered with a layer of albolene oil to prevent the escape of $CO_2$ and subsequent change in reaction during shaking. At the end of the period of shaking the solid was removed by centrifuging, and determinations of Ca, P, and pH were made upon the supernatant fluid. $PO_4^{\equiv}$ concentrations and stoichiometric solubility products for tertiary calcium phosphate $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$ were calculated as described in a previous paper,[1] and were compared with ion products calculated from analyses of the same specimens of spinal fluid before shaking with $Ca_3(PO_4)_2$.

*Calcium.*—Determinations were made by the micro method of Kramer and Tisdall (2).

[1] Holt, L. E., Jr., La Mer, V. K., and Chown, H. B. (1), p. 518.

*Phosphorus.*—Determinations were made by the Briggs modification (3) of the Bell-Doisy method.

*Hydrogen Ion.*—Determinations were made colorimetrically by means of buffer standards which had recently been checked electrometrically.

*Spinal fluids.*—Specimens A and C were pooled spinal fluids, some of them normal and others luetic. These fluids were not collected under albolene, and $CO_2$ had therefore escaped. Before setting up the experiment the fluids were covered with albolene, and the reaction was adjusted to pH 7.4 by passing in $CO_2$ gas. Preliminary samples were then taken for analysis and the $Ca_3(PO_4)_2$ was introduced, after which the shaking was commenced. Although sterile precautions were not maintained in these two experiments, the bacterial growth was slight.

Specimen B consisted of two normal spinal fluids collected under albolene. Sterile conditions were maintained throughout this experiment.

Analyses of the fluids before and after shaking with $Ca_3(PO_4)_2$ and calculations of the product $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$ are given in Table I.

*Comment.*

From the figures given in Table I it can be seen that shaking with $Ca_3(PO_4)_2$ causes a diminution in the Ca and P content of the fluid, and also a decrease in the value of the ion product $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$. This latter falls from an average of $1.5 \times 10^{-26}$ (p product = 25.83) to less than $1.0 \times 10^{-28}$ (p product > 28.00). The decrease in the ion product is so striking as to leave little doubt that *cerebrospinal fluid, like blood serum, is normally very much supersaturated*[2] *with* $Ca_3(PO_4)_2$. Although the actual solubility of $Ca_3(PO_4)_2$ in spinal fluid could not be accurately determined, since the final concentrations of Ca and P were often beyond the limits of analytical accuracy, still it is quite clear that *this salt is much less soluble in spinal fluid than in blood serum.* In serum the $K_{s.p.}$ of $Ca_3(PO_4)_2$, expressed in stoichiometric units, was found (1) to be about $10^{-26}$, while the figures given above indicate that the $K_{s.p.}$ in spinal fluid is smaller than $10^{-28}$. The decreased solubility of this salt in spinal fluid is just what would

[2] At least 200 per cent in terms of molar solubility.

TABLE I.

| | pH | Calcium. | | Phosphorus. | | $[PO_4^{\equiv}]$ | $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$ | - log product (p product). |
|---|---|---|---|---|---|---|---|---|
| | | *mg. per 100 cc.* | *mols.* | *mg. per 100 cc.* | *mols.* | *mols.* | | |
| Specimen A. | | | | | | | | |
| Before shaking.. | 7.4 | 5.2 | $1.3 \times 10^{-3}$ | 1.3 | $4.2 \times 10^{-4}$ | $3.0 \times 10^{-9}$ | $2.0 \times 10^{-26}$ | 25.70 |
| After shaking 9 days..... | 7.4 | <1.0 | $<2.5 \times 10^{-4}$ | <0.5 | $<1.6 \times 10^{-4}$ | $<1.1 \times 10^{-9}$ | $<2.1 \times 10^{-29}$ | >28.69 |
| Specimen B. | | | | | | | | |
| Before shaking.. | 7.4 | 4.5 | $1.1 \times 10^{-3}$ | 1.3 | $4.2 \times 10^{-4}$ | $3.0 \times 10^{-9}$ | $1.3 \times 10^{-26}$ | 25.88 |
| After shaking 8 days..... | 7.3 | 1.5 | $3.75 \times 10^{-4}$ | <0.5 | $<1.6 \times 10^{-4}$ | $<8.5 \times 10^{-10}$ | $<3.8 \times 10^{-29}$ | >28.42 |
| Specimen C. | | | | | | | | |
| Before shaking.. | 7.4 | 4.7 | $1.2 \times 10^{-3}$ | 1.2 | $3.9 \times 10^{-4}$ | $2.8 \times 10^{-9}$ | $1.3 \times 10^{-26}$ | 25.90 |
| After shaking 7 days..... | 7.65 | 2.5 | $6.25 \times 10^{-4}$ | <0.5 | $<1.6 \times 10^{-4}$ | $<2.3 \times 10^{-9}$ | $<1.3 \times 10^{-27}$ | >26.88 |

Average p product before shaking = 25.83.
" " " after " = 28.00+.

be expected[3] from the theory of solubility in salt solutions developed by Brönsted and by Debye and Hückel.

It seems only logical to relate the smaller solubility of $Ca_3(PO_4)_2$ in spinal fluid to the lower concentrations of Ca and P normally found there (see Table II). It has been our experience with both inorganic and biological fluids that, in the absence of the solid salt, a state of supersaturation with $Ca_3(PO_4)_2$ to the extent of 200 to 250 per cent may continue almost indefinitely. Higher degrees of supersaturation, however, cause comparatively rapid deposition of the salt. It therefore seems likely that both serum and spinal fluid are normally supersaturated nearly to the maximum extent which is stable, and it is probably on this account that the concentrations in spinal fluid are no higher. There is

TABLE II.

*Concentration of Cations and Anions in Normal Adult Serum and Spinal Fluid.*

Taken from data of Pincus and Kramer (5).

| Material. | Na | K | Ca | Cl | $HCO_3$ | Total $PO_4$ |
|---|---|---|---|---|---|---|
| | *mols* | *mols* | *mols* | *mols* | *mols* | *mols* |
| Serum..... | 0.1426 | 0.0053 | 0.0024 | 0.0989 | 0.025 | 0.0009 |
| Spinal fluid.... | 0.1526 | 0.0038 | 0.0012 | 0.1218 | 0.025 | 0.0004 |

thus an intimate relation between the solubility of $Ca_3(PO_4)_2$ and the concentrations of Ca and P which are stable in either medium.

The existence of factors like the parathyroid hormone, the active principle of cod liver oil and ultra-violet radiation, which may presumably influence the degree of supersaturation, should not be forgotten in this connection. Under physiological conditions, however, such factors should exert a constant influence, and one which is probably identical in the two fluids; hence the difference in the concentrations of Ca and P cannot be attributed to them.

The nature of the relation between blood serum and cerebrospinal fluid has always been a perplexing problem. Although some observers (4) have not hesitated to make the bold assumption that spinal fluid is merely a dialysate of serum, it has been difficult to explain the unequal distribution of the inorganic electrolytes on this basis. Recently Pincus and Kramer (5) have suggested that the Donnan equilibrium plays an important part in this connec-

[3] This is more fully discussed in a previous paper, (1).

tion, and they have made calculations to show that at least some of the criteria for the existence of a Donnan equilibrium are fulfilled. It is, however, clear that not all the inequalities in the distribution of the inorganic electrolytes can be explained on this basis. It can be seen from the figures in Table II that the differences in the concentrations of the calcium and phosphate ions in particular are much greater than would be demanded by a simple Donnan equilibrium.[4] Moreover, the differences are not always in the right direction. Thus from the Donnan equilibrium one would expect to find higher concentrations of anions in the spinal fluid. The concentration of phosphorus, and hence of the various phosphate ions, is, however, only about half as great as in serum.

On the other hand it is quite possible to explain these variations on the basis of the electrostatic forces between the ions in these two systems. In the blood serum, a medium in which the polyvalent protein ions play a conspicuous part, the activity of the calcium and the phosphate ions is restricted, and the activity coefficients of these ions are therefore much smaller than in a medium like spinal fluid. Thus the stoichiometric concentrations may be very much higher in serum, although the activities of these ions are not greatly different from those in the spinal fluid.[5]

We have then two known physicochemical factors which can account for the unequal distribution of the inorganic electrolytes between blood serum and cerebrospinal fluid: (1) the Donnan equilibrium, which causes an inequality of the ion activities in the two systems, due to the fact that the protein ions cannot diffuse into the spinal fluid, and (2) the electrostatic effects of the polyvalent protein ions of the serum, which diminish the activity coefficients of the inorganic ions there, thus making it possible for a high stoichiometric concentration to coexist with a comparatively low activity. As would be expected, it is with the di- and trivalent ions that this effect is particularly striking.

There are, however, certain inequalities in the distribution of the various ions which cannot be accounted for by either of the

[4] Calculated on the basis of the combining power of the serum proteins, as determined by Van Slyke, Wu, and McLean (6).

[5] Even at equilibrium the activities of these ions should not be exactly equal in the two media, because of the small inequalities demanded by the Donnan equilibrium, which concerns activities, the true thermodynamic units.

above factors, as, for instance, the higher concentration of sodium in the spinal fluid. While it is altogether possible that all such facts may eventually be explained on the basis of a physico-chemical equilibrium, for the present it would seem best to wait until such a time before concluding that cerebrospinal fluid is a dialysate rather than a true secretion.

## SUMMARY.

1. $Ca_3(PO_4)_2$ is far less soluble in cerebrospinal fluid than in blood serum.

2. Cerebrospinal fluid, like blood serum, is normally supersaturated with $Ca_3(PO_4)_2$.

3. The greater solubility of $Ca_3(PO_4)_2$ in blood serum and the higher concentrations of calcium and phosphate ions in blood serum can be explained by the electrostatic effects involving the activities of these ions.

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# THE DETERMINATION OF CYSTINE BY MEANS OF FEEDING EXPERIMENTS.*

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(Received for publication, July 18, 1925.)

The early experiments of Osborne and Mendel showed very clearly that growth in young laboratory animals (rats) could be limited by the lack of a single amino acid. By means of feeding experiments these workers, and others by the use of similar methods, have determined the qualitative limitations of a number of purified proteins and of certain natural food materials.

If under properly controlled experimental conditions, an increase in the growth rate can be induced by the addition of a single amino acid to the diet, as definitely shown by Osborne and Mendel in April, 1914[1] and several subsequent papers, it seems that this may be interpreted to indicate that there is an unused margin in the basal diet of all other essential amino acids. With such a diet, then, it is reasonable to anticipate that different additions (below the optimum) of the limiting amino acid would give correspondingly different responses in growth within the limits set by the second limiting amino acid in the basal diet.

If this graded response in growth could be measured quantitatively for known additions of the limiting amino acid to a given basal diet, it should become possible to use such methods and data in estimating the amount of the definite amino acid supplied by (contained in) any given protein which added in known amounts to the same basal diet gives a like increase in the rate of growth.

Early in 1916, Osborne and Mendel[2] published data showing differences in growth response to different quantitative additions

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[1] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1914, xvii, 325.

[2] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1916, xxv, 1.

of lysine and tryptophane, and in later papers they have referred further to the quantities of certain amino acid additions required to induce a normal or standard rate of growth in the experimental animal.

In the investigation reported in this paper, we have undertaken to apply the method of feeding experiments to the quantitative determination of cystine. Using pure cystine as the basis for the establishment of the quantitative relationships, we have chosen casein as the first material to which to apply the method because Osborne and Mendel[3] have shown that cystine while present in casein, is the first amino acid to be brought below the amount required for normal growth when casein is fed as sole protein and in insufficient percentage of the food mixture. This gives importance from the standpoint of the chemistry of nutrition to the quantitative proportion of cystine in casein, while attempts to determine the cystine content of casein quantitatively *in vitro* have yielded extremely variable results.

Following Osborne and Mendel's demonstration that cystine is the first "limiting" amino acid of casein, it was shown in this laboratory[4] that cystine is also the first limiting amino acid of the proteins of whole milk—or at least of a basal diet in which whole milk furnished all of the protein except the small amount contained in the yeast used as a supplementary source of vitamin B. Using, then, a basal diet whose protein content was of this character, it seemed possible to establish quantitatively the responses in growth induced by the addition of known small proportions of cystine to the diet. If, further, we could establish the amounts of added casein required to induce the same increase in growth as a known addition of pure cystine, these data should be applicable to the estimation of the cystine content of the casein thus fed.

## EXPERIMENTAL.

*General Method.*—The plan was to compare the gains in weight during an experimental period of 6 weeks, made by standard young animals on a basal diet of limited cystine content but adequate for normal growth in all other respects, with the gains made by strictly comparable animals on the same diet supplemented by

[3] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 351.
[4] Sherman, H. C., and Merrill, A. T., *J. Biol. Chem.*, 1925, lxiii, 331.

known additions of pure cystine (or by known additions of casein, whose cystine content was to be thus determined).

The basal diet as used in this study had the following composition (Diet 324).

| | per cent |
|---|---|
| Whole milk powder | 16.70 |
| Cod liver oil | 1.00 |
| Dried brewers' yeast | 1.65 |
| Salt mixture[8] | 1.65 |
| Starch | 79.00 |

When casein was introduced into this diet, it replaced an equal amount of the starch.

The diet, whether with or without the cystine or casein supplement, was well relished by the experimental animals.

These were healthy young albino rats of a uniform initial age of 28 to 29 days, taken from families of known nutritional history and so arranged that direct comparisons were made between litter mates so as to minimize the factor of physiological variability. This was also further controlled by numerous repetitions of these direct comparisons. With one exception, the data appearing in the tables and chart which follow are averages of the records of from 8 to 21 animals, each in direct comparison with one or more litter controls. In our judgment both large numbers and litter controls should be emphasized if average data of the highest accuracy and validity are to be obtained.

The adequacy of the basal diet and the suitability of the experimental animals used in these experiments have been established for the conditions and the materials employed by us in a much larger experience than could well be described in detail here.

*Effects of Added Cystine.*—The experiments upon the effects of added cystine were carried out in three series somewhat separated as to time and in the case of the last series differing also in the diets of the families from which the young animals for experimental purposes were drawn. The experiments of the first series were carried out during the spring and summer of 1923 and all the experimental animals were drawn from families on the same diet (Diet D) which was composed of two-thirds whole milk powder and one-third ground whole wheat with table salt added in the

[8] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

proportion of 2 per cent of the weight of the wheat. The experiments of the second series were performed during the spring of 1924 with animals from the same family diet as in the first series. The rate of growth on the basal diet was higher in the second series than in the first, but the increased growth resulting from a given addition of cystine was essentially alike in the two series of experiments. For the experiments of the third series, carried out in the fall of 1924 and winter of 1924–1925, only a few animals from Diet D (from which those used in the first and second series were drawn) were available. The experimental animals used in this third series were therefore drawn chiefly from families on our Diets A

TABLE I.

*Increased Gains in 6 Weeks due to Graded Additions of Cystine to Basal Diet (Diet 324).*

| Proportion of cystine added. | Increased gain in 6 weeks. | No. of experimental animals. |
|---|---|---|
| *per cent* | *gm.* | |
| 0.01 | 5.4 | 2 |
| 0.025 | 10.0 | 17 |
| 0.05 | 17.9 | 12 |
| 0.10 | 20.6 | 21 |
| 0.20 | 28.6 | 8 |

and B which have been described in a previous paper from this laboratory.[6] It was found that the gains made on the basal diet alone were in both cases much like those obtained with rats from Diet D in the experiments of the second series. As regards response to the added cystine the animals from Diet B reacted essentially like those from Diet D as used in the first and second series, while those from Diet A showed an even more marked response. It happened that the use of the animals from families on Diet A was confined to the experiments dealing with the very smallest additions of cystine (1 part of added cystine to 10,000 parts of the basal diet, or 0.01 per cent). Hence in order to bring out the quantitative relationship of the responses to different cystine additions the experiments with rats from Diet A are not included in the general averages summarized in Table I and Fig. 1; but it will be seen that the experience with rats from Diet A suggests

[6] Sherman, H. C., and Campbell, H. L., *J. Biol. Chem.*, 1924, lx, 5.

the possibility of finding conditions under which feeding experiments may be made an even more delicate method of testing for cystine than is claimed in what follows.

When the results of all the experiments upon rats from Diets

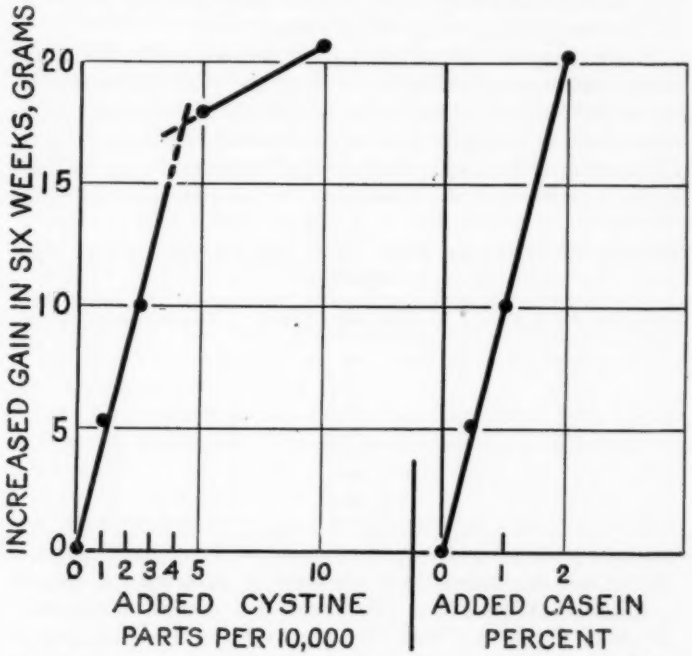


FIG. 1. Rate of gain in weight as related to quantitatively graded additions of cystine or of casein to the same basal diet. In these experiments casein appeared to furnish one-fortieth of its weight of cystine. The region in which cystine is the limiting factor and response in growth is directly proportional to cystine added is established to 0.025 per cent added cystine with 10 gm. added gain and appears to extend to an addition of 0.04 per cent of cystine or to an increased gain of about 17 gm. in an experimental period of 6 weeks.

B and D (and slight modifications of these diets) are combined and the increased gains due to cystine additions are plotted against the proportions of cystine added, as in Fig. 1, it appears that the rate of growth rises rapidly with small additions of cystine (to the basal

diet here used) and that the relationship is essentially arithmetical (or the curve of increase linear) up to an addition of at least 0.025 per cent and probably up to 0.04 per cent cystine, with resulting increased gains of 10 gm. or 17 gm. respectively in 6 weeks. The responses to larger additions of cystine (for this basal diet increments above 0.05 per cent) are at a lower rate.

It would appear that at least within the limits of the first linear region interpolation should be accurate and it should be possible to use the feeding method as here developed for the quantitative determination of cystine with very satisfactory results, the degree of precision of the results thus obtained depending upon the number of repetitions of the experiment for the determination of any

TABLE II.

*Increased Gains in 6 Weeks Due to Graded Additions of Casein to Basal Diet (Diet 324).*

| Proportion of casein added. | Increased gain in 6 weeks. | No. of experimental animals. |
|---|---|---|
| *per cent* | *gm.* | |
| 0.47 | 5.0 | 13 |
| 1.0 | 10.2 | 13 |
| 2.0 | 20.3 | 16 |
| 3.5 | 31.5 | 20 |
| 8.0 | 49.2 | 9 |
| 16.0 | 90.3 | 9 |

one ratio, the adequacy of the control of the experimental conditions, and the uniformity of the stock of experimental animals.

*Effects of Added Casein.*—In the first and third of the series described above, rats from the same litters as those used for the study of pure cystine additions were given casein supplements with the same basal diet.

As little as 0.3 per cent of added casein resulted in distinctly increased gain. In Table II the results of experiments with 0.3 to 0.5 per cent casein are averaged together, in order to minimize the effects of individual variability, and appear as an average increase of 5.0 gm. resulting from an average addition of 0.47 per cent casein. Similarly the results of experiments with 3 per cent and with 4 per cent casein are averaged together.

The average results are summarized in Table II and the second curve of Fig 1.

A comparison of these results with those obtained by the addition of pure cystine to the same basal diet shows a close parallelism in the early parts of the cystine and casein curves, indicating that within this range of increased gain, the casein probably served essentially as a source of cystine, the other essential amino acids being supplied in sufficient quantities for this rate of growth by the basal diet. Beyond this point further additions of casein give relatively greater returns in the growth rate than do corresponding amounts of pure cystine, probably because beyond a certain point in the gain induced by cystine, some other amino acid which casein supplies is beginning to be a determining ("limiting") factor.

These considerations suggest that comparisons of the growth responses induced by additions of cystine and casein respectively, if they are to be interpreted as indications of the cystine content of the casein, should be confined to cases in which the increase of growth during the 6 weeks experimental period is not over 10 to 17 gm. above the gain made on the basal diet, within which limits both curves are plainly linear.

From the averages of our comparable experiments with graded feedings of cystine and of casein respectively as supplements to the same basal diet, as summarized in Tables I and II and Fig. 1, it is plain that essentially equal gains were induced by 1 per cent casein and by 0.025 per cent cystine, indicating that casein contains 2.5 per cent of cystine. From other experiments in which, as explained above, the responses to small amounts of pure cystine were greater, estimates as low as 1.3 to 1.5 per cent of cystine in casein may be made.

## SUMMARY AND DISCUSSION.

A basal diet is described in which cystine is the growth-limiting factor.

When carefully standardized young rats are fed this basal diet, with and without graded additions of cystine, it is possible to determine a direct linear or arithmetical relationshib petween the added cystine fed and the added growth thereby induced, up to an added gain of at least 10 gm. in an experimental period of 6 weeks, induced by the addition of 0.025 per cent of cystine to the basal diet, and probably up to 0.04 per cent added cystine with an added gain of 17 gm.

Parallel experiments with graded additions of casein to the same basal diet gave correspondingly graded responses in the growth rate.

Within the range of growth increase in which the response to added cystine was in arithmetical proportion to its amount, it is believed that the response to the added casein may be interpreted as a measure of the cystine (or cystine plus nutritionally equivalent sulfur-containing radicles) furnished by the casein.

Thus interpreted, our results would indicate that casein contains not less than 1.3 per cent nor more than 2.5 per cent of cystine, or that approximately three-eighths to three-fourths of the total sulfur in casein is in the form of cystine or of cystine plus other sulfur-containing radicles which are interchangeable with cystine in nutrition.

The lower limit of the range of possible percentage of cystine in casein thus indicated by these feeding experiments is higher than the proportion of cystine obtained from casein *in vitro* by present analytical methods.

We believe that the method here developed for the determination of cystine by means of feeding experiments is quantitative in principle within the limits of growth increase which we have described, and that while natural variability must limit the precision of individual experiments, the accuracy of mean results can be carried to practically any degree which the importance of the problem may justify, by the performance of large numbers of strictly comparable experiments and statistical interpretation of the data thus obtained.

With the development of a suitable basal diet for each, it should be possible to apply the same general principle to the quantitative determination of other nutritionally essential amino acids. Whether such feeding experiments, or analytical procedures *in vitro*, will yield the more accurate indication of the true amount of any particular amino acid in an isolated protein, or in a natural food material, can only be determined by thorough investigation of each case or type of cases. The method of determination by means of feeding experiments may perhaps be expected to be of most value in the cases of cystine and tryptophane, whose nutritional importance is great and whose quantitative isolation *in vitro* has proven especially difficult.

# THE IMMUNOLOGICAL PROPERTIES OF ALCOHOL-SOLUBLE VEGETABLE PROTEINS.

## IX. THE BIOLOGICAL REACTIONS OF THE VEGETABLE PROTEINS.

By JULIAN H. LEWIS and H. GIDEON WELLS.

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(Received for publication, July 20, 1925.)

Numerous alcohol-soluble vegetable proteins, prepared in the laboratory of T. B. Osborne, have previously been studied in respect to their immunological activity and specificity.[1] These investigations concerned chiefly gliadin from wheat and from rye, hordein from barley, and zein from maize, but some tests were made with the alcohol-soluble proteins from oats and from sorghum seeds. These proteins were found to be antigenic in such degree as their solubility in the body fluids permitted, so that a fair proportion of severe and fatal anaphylactic reactions could be obtained, even by intraperitoneal injections into sensitized guinea pigs with doses as small as 5 mg. The gliadins from wheat and rye were found to be similar, and apparently identical, by anaphylactic tests, as they seem to be chemically. Hordein was found immunologically related to the gliadins of wheat and rye, but distinguishable from both by saturation tests. Zein was found to be distinct from gliadin and from hordein.

Through the kindness of Professor R. A. Gortner of the University of Minnesota, we have been given the opportunity to study the immunological reactions of another series of alcohol-soluble vegetable proteins, which have been isolated and studied chemi-

[1] Wells, H. G., and Osborne, T. B., *J. Infect. Dis.*, 1911, viii, 66; 1913, xii, 341; 1914, xiv, 377; 1915, xvii, 259. Lake, G. C., Osborne, T. B., and Wells, H. G., *J. Infect. Dis.*, 1914, xiv, 364. Elsesser, O. J., *J. Infect. Dis.*, 1916, xix, 655.

cally by him and W. F. Hoffmann.[2] These prolamines were isolated from wheat, *Triticum vulgare*; durum, *Triticum durum*; emmer, *Triticum dicoccum*; einkorn, *Triticum monococcum*; rye, *Secale coreale*; oats, *Avena sativa*; barley, *Hordeum vulgare*; spelt, *Triticum spelta*; corn, *Zea mays*; kafir, *Andropogon sorghum*; teosinte, *Euchlaena mexicana Schrad.*; and sorghum, *Sorghum vulgare*. The chemical properties, as known from such data as are contained in the literature and those obtained by Gortner and Hoffmann, indicate that these proteins may be divided into a "wheat group," which includes the proteins isolated from the genus *Triticum*, and a "corn group," including those isolated from maize, teosinte, kafir, and sorghum. According to these authors the genetic behavior of these cereals has been extensively studied by plant breeders, although relatively more work has been done upon the wheat group. It has been shown that *Triticum monococcum* is characterized by having seven chromosomes, that *Triticum dicoccum* and *Triticum durum* have fourteen chromosomes, and *Triticum vulgare* and *Triticum spelta* have twenty-one chromosomes, as if their evolution had been accomplished by polymerization. The fertility or total or partial sterility of interspecies is said to be in accordance with these chromosome numbers. In the "corn group" it has been found that teosinte will hybridize with *Zea mays*, indicating a genetic relationship.

Because of these interesting genetic relationships, as well as on account of the known chemical similarities and differences between the various members of these two groups, their immunological relationships are of particular significance, and hence we have carried out the following studies with the complement fixation reaction and by different methods of determining anaphylaxis reactions. Our results are summarized briefly below.

### *The Complement Fixation Test.*

In order to study the relation of these proteins to each other with the complement fixation test, three type antisera were pre-

[2] The chemical and physicochemical data on these proteins appear in a report by Gortner, R. A., and Hoffmann, W. F., in the Second colloid symposium monographs, Chemical Catalogue Co., New York, 1925. A preliminary report on both the immunological and chemical observations has been published by Lewis, J. H., Wells, H. G., Hoffmann, W. F., and Gortner, R. A., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 185.

pared by immunizing rabbits with gliadin, the alcohol-soluble protein of wheat; zein, the alcohol-soluble protein from corn; and glutenin, also derived from wheat but chemically dissimilar to gliadin. In immunizing the rabbits it was necessary to give many doses of the proteins because of their low solubility, which limited the amount of protein that could be given in the maximum volume of fluid safe to inject. 50 mg. of the protein were ground in a mortar with 5 cc. of 0.2 per cent NaOH, and then 5 cc. of water added. The undissolved protein was allowed to settle out and the supernatant fluid injected subcutaneously. These injections were repeated every 4 days until about 15 were given. Such a procedure produced fairly potent antisera. 10 days after the last injection the animals were bled from the ear veins and the serum obtained was heated to 56° for ½ hour. It was used as soon thereafter as possible, to avoid the development of anti-complementary action. Each of the three antisera was tested against the remaining proteins of both the wheat and corn series as well as the homologous proteins. The amount of antiserum used was 0.2 cc. and the antigen used in amounts of 1.0, 0.1, and 0.01 mg. Two units of complement were added and fixation incubation carried on in the ice box for 14 hours. The hemolytic system consisted of washed sheep cells and amboceptor. The final incubation was carried on in the water bath for ½ hour and the results read immediately. In Table I the results are given. They indicate that the optimal amount of antigen for differentiation of the proteins was 0.01 mg. With this amount the alcohol-soluble proteins of the wheat group (consisting of gliadin, emmer, durum, spelt, and einkorn) and the alcohol-soluble proteins of the corn group (consisting of zein, kafirin, and teosinte) are sharply distinguishable. With the larger amounts the wheat series have a tendency to react alike with antiglutenin and antigliadin sera, but with 0.01 mg. amounts a differentiation is obtained between glutenin and gliadin and other members of the so called wheat group of alcohol-soluble proteins. Probably with still smaller amounts of antigen a difference between the individual members of each group could be detected and the degree of relation to the antigen used to produce the antiserum could be shown.

TABLE I.

| | Antigliadin. | | | Antiglutenin. | | | Antizein. | | | Control. | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 mg. | 0.1 mg. | 0.01 mg. | 1 mg. | 0.1 mg. | 0.01 mg. | 1 mg. | 0.1 mg. | 0.01 mg. | 1 mg. | 0.1 mg. | 0.01 mg. |
| Gliadin | ++++ | ++++ | ++++ | ++++ | +++ | ++ | 0 | 0 | 0 | 0 | 0 | 0 |
| Glutenin | ++++ | 0 | 0 | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |
| Emmer | ++++ | ++++ | ++++ | ++++ | ± | ± | 0 | 0 | 0 | 0 | 0 | 0 |
| Durum | ++++ | ++++ | ++++ | ++++ | 0 | 0 | +++ | 0 | 0 | 0 | 0 | 0 |
| Spelt | ++++ | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Einkorn | ++++ | ++++ | ++++ | ++++ | + | + | 0 | 0 | 0 | 0 | 0 | 0 |
| Teosinte | ++ | 0 | 0 | 0 | 0 | 0 | ++++ | ++++ | ++++ | ++ | 0 | 0 |
| Zein | 0 | 0 | 0 | 0 | 0 | 0 | ++++ | ++++ | ++++ | 0 | 0 | 0 |
| Kafirin | 0 | 0 | 0 | 0 | 0 | 0 | ++++ | ++++ | ++++ | 0 | 0 | 0 |

*Anaphylaxis Reactions.*

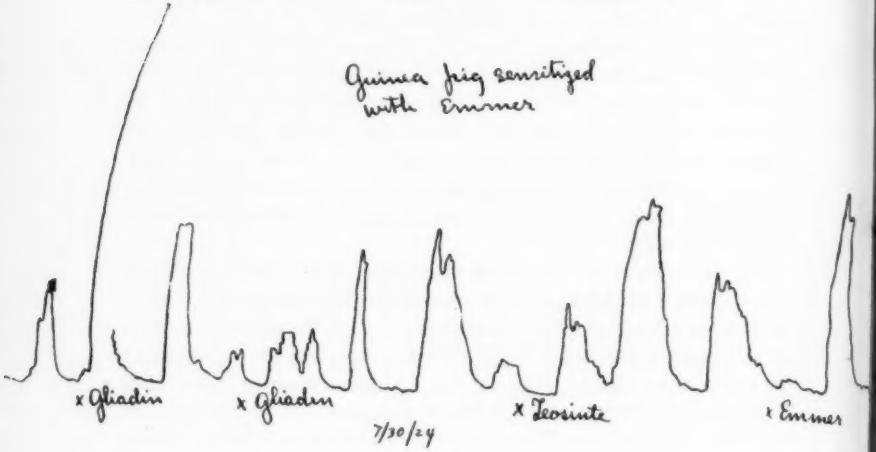
The results obtained with the complement fixation test were confirmed by a series of studies carried out with the anaphylaxis reaction, by means of three different methods.

*Uterus Strip Method.*—The uterus strip method of Dale is very well adapted to show the relation of proteins. A uterus sensitized to one protein will show a contraction to another protein of chemical similarity, and two chemically similar proteins will equally desensitize a uterus that is sensitive to either one. Thus, a series of proteins, such as we are studying, can be arranged according to their similarity by studying their interactions with the uterine strip.

For this purpose, two guinea pigs were sensitized with small doses of each of these proteins, and after an incubation period of 14 days or more they were killed and a horn of the uterus mounted in the Dale apparatus. To the Locke's solution in the chamber containing the uterus strip, 10 mg. of one of the proteins other than that to which it was sensitized, was added. If there was no response, the strip was washed and another protein added. When a response was obtained, the active protein was added a second time, after washing, in order to desensitize the strip completely. When completely desensitized to the heterologous protein, the homologous protein was added to see if desensitization had also been obtained to this. The second horn of the same uterus was then mounted and tested in a similar manner, but with another combination of proteins. Thus many cross-reactions were studied, which showed the ability of one protein to sensitize to another or the ability of a heterologous protein to desensitize a uterine strip to the homologous protein.

The results showed, as did the complement fixation tests, that the proteins of the wheat group were separate and distinct from the corn group proteins, in that a uterine strip that is sensitive to one of the former was never sensitive to any of the latter, or *vice versa*. They also showed that, by this method, the members of one group are indistinguishable from each other, because a uterus sensitive to one member is likewise sensitive to any other member of the same group, and after a contraction is obtained it is desensitized not only to all other members of the group but also to the one with which it was sensitized.

Curve 1 shows a tracing of a uterus strip from a guinea pig sensitized with emmer. A marked reaction is obtained with gliadin, but not, thereafter, with teosinte and emmer.

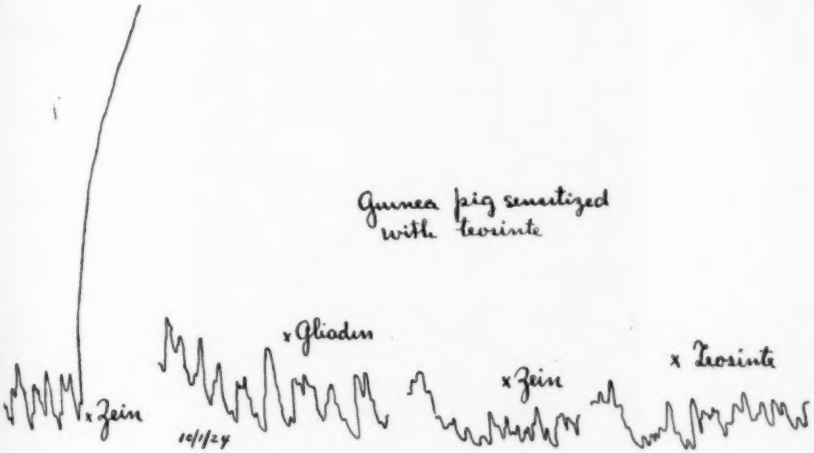



CURVE 1.

CURVE 2.

Curve 2 is of a uterus from a guinea pig sensitized with zein. No reaction is obtained with gliadin, spelt, and durum, but it does react with teosinte and is, thereafter, not sensitive to kafirin.

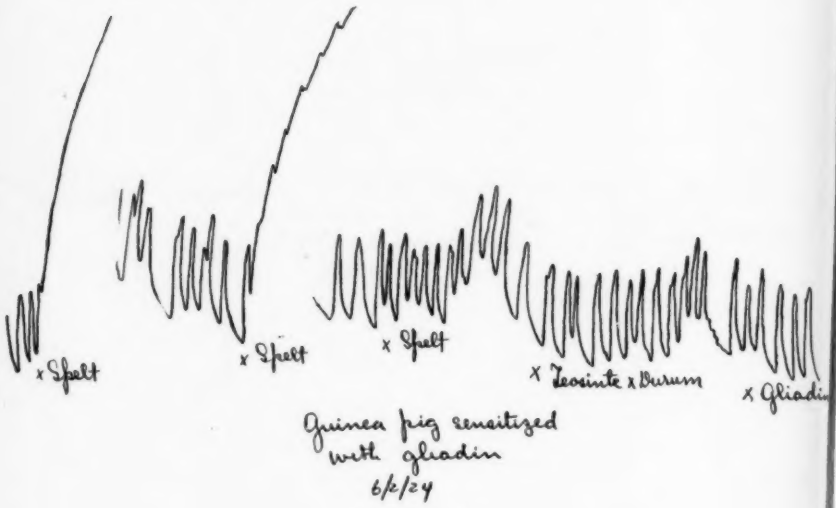
CURVE 3.



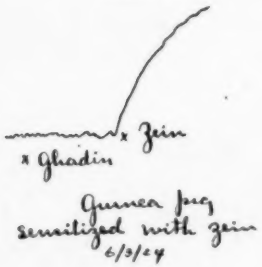

CURVE 4.

Curve 3 was obtained with a uterus strip sensitized with gliadin. It reacts with emmer, after which it does not react with zein, durum, or the homologous protein, gliadin.

Curve 4 is of a uterus sensitized with teosinte. A reaction is obtained with zein, but not with gliadin, zein, or teosinte, which were tested in this order.



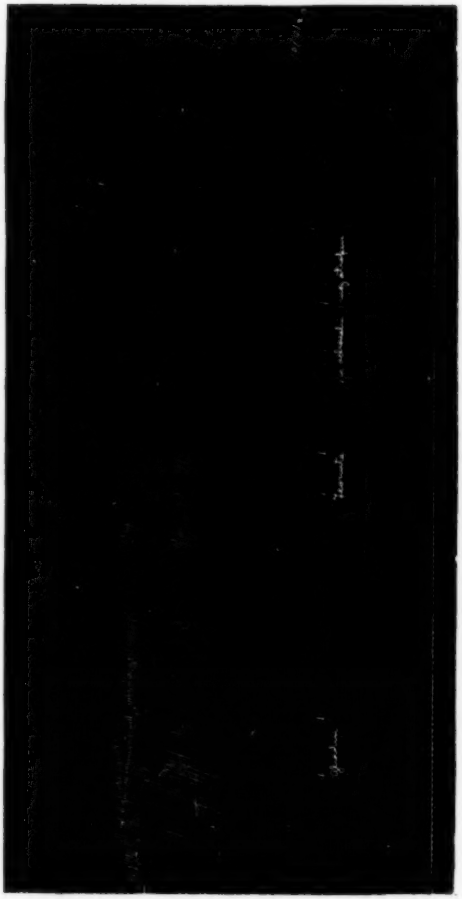

CURVE 5.



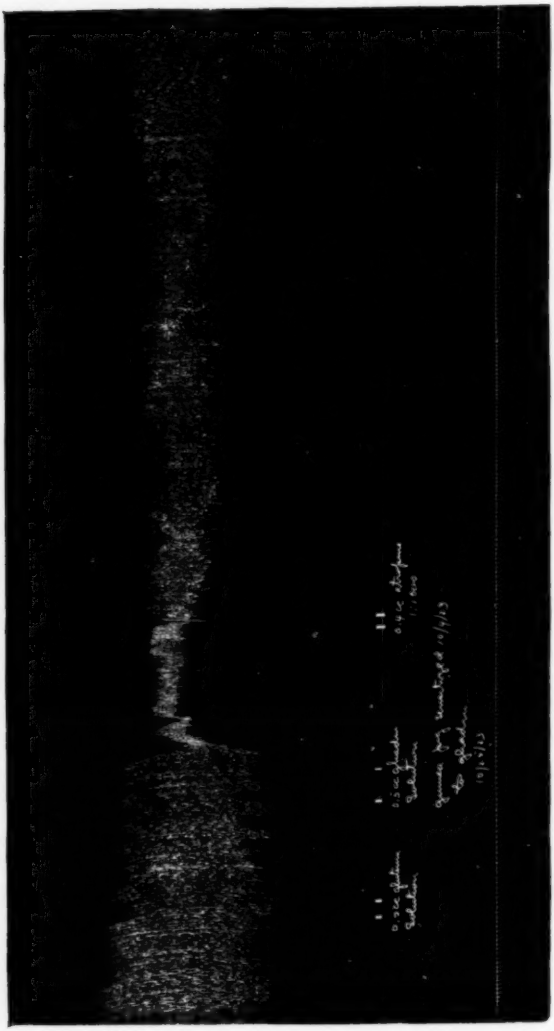

CURVE 6.

Curve 5 represents a uterus from a guinea pig sensitized with gliadin. When finally desensitized to spelt, to which it gave two strong reactions, it does not react to teosinte, durum, or gliadin.

Curve 6 shows that a uterus strip sensitized to zein does not react with gliadin, which also fails to desensitize it to zein.



CURVE 7.



CURVE 8.

*Bronchospasm Method.*—This depends on the fact that, as pointed out by Auer and Paul Lewis, in guinea pigs the most striking effect in anaphylactic shock is the spasm of the bronchial musculature, which causes complete occlusion of the bronchi in severe reactions. A method for securing tracings showing the degree of obstruction of the bronchi in the intact lung has been devised by Koessler and Lewis,[3] which was used with these prolamines. The results obtained confirmed those obtained with the uterine strip method.

Curve 7 shows the response of the bronchial musculature of a guinea pig sensitized with zein,when first gliadin and then teosinte are injected. Gliadin has no effect while teosinte causes a marked bronchospasm.

Curve 8 shows the response to glutenin and gliadin in an animal sensitized to gliadin. Glutenin has no effect, while gliadin causes a bronchospasm that is relieved with atropine.

*Anaphylactic Shock in Guinea Pigs.*—In these experiments, 250 gm. guinea pigs were sensitized with wheat gliadin or zein, and then after 2 weeks given intraperitoneal injections of the different prolamines. Although this method is not so delicate as the uterus strip and bronchospasm methods, especially with proteins having such a low solubility in water and weak salt solutions, the results were quite the same, showing practical identity of proteins in the two groups. That is, guinea pigs sensitized with gliadin gave strong reactions when reinjected with the other four proteins of the wheat series, but none at all with proteins from the corn series. Animals recovering from these crossed reactions were found to be protected to gliadin just as were the tissues in the uterine strip experiments.

The solubility of the zein and teosinte being particularly poor, the reactions with intraperitoneal injections were not ordinarily severe enough to be conclusive as to identity. With intracardiac injections of either the homologous or the heterologous protein in sensitized animals, however, severe fatal reactions were readily obtained. A series of guinea pigs was then sensitized with zein and teosinte, and desensitized by being given 100 mg. doses of the heterologous protein intraperitoneally. 48 hours later those animals failed to react when given either the homologous or the

[3] Koessler, K. K., and Lewis, J. O. (in press).

heterologous protein by the intracardiac route, indicating that the two proteins are similar enough to one another to desensitize sensitized animals.

## CONCLUSIONS.

Immunological study of the alcohol-soluble proteins from certain cereal grains indicates that they fall into two classes. Gliadin from wheat and rye and the prolamines from durum, einkorn, emmer, and spelt, of the genus *Triticum*, seem to be very closely related, according to tests by means of both the complement fixation and the anaphylaxis reaction (tested by three methods). The alcohol-soluble proteins from common maize, from kafir corn, and from teosinte, were found to be closely related to one another, but not to the alcohol-soluble proteins of the *Triticum* group. These results agree with observations made by others (Gortner and Hoffmann) that the chemical properties of these alcohol-soluble proteins indicate the existence of a "wheat group" and a "corn group" of prolamines, the members of each group being chemically similar to each other, as we have found them to be immunologically.

# THE EFFECT OF FERMENTATION WITH SPECIFIC MICRO-ORGANISMS ON THE VITAMIN C CONTENT OF ORANGE AND TOMATO JUICE.*

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It has already been pointed out that both corn silage and sauerkraut are free from vitamin C (1). Silage can be considered as preserved green corn, yet it has been found devoid of vitamin C even when prepared under carefully controlled laboratory conditions where the temperature was not allowed to rise appreciably above room temperature. Green corn itself is a very effective antiscorbutic material—1 gm. of the green material fed daily (2) being sufficient to protect the guinea pig from scurvy, while 1.5 gm. of fresh cabbage will do the same thing (3). In the case of silage making it would seem unlikely that the destruction of vitamin C was due to oxidation because large quantities of carbon dioxide are produced by the biological and respiratory processes going on in silage making. The same situation is true in sauerkraut making where there is a very rapid production of carbon dioxide and displacement of a large part of the oxygen in the interstices of the mass.

It was believed possible that microorganisms were instrumental in the destruction of vitamin C although it was also considered possible that the destruction was due to direct oxidation, the oxygen not being completely used up or displaced during the initial fermentation processes of silage or sauerkraut making.

* Published with the permission of the Director of the Wisconsin Agricultural Station, Madison.

Further, in studying this problem we had in mind the possible use of organisms as agents in removing sugars and other carbohydrates from materials rich in vitamin C as a means of studying and purifying this substance. This part of the field has been particularly developed by Zilva (4) in his work on vitamin C preparations, through the use of yeast fermentations.

## EXPERIMENTAL.

Before discussing the effect of fermentation by microorganisms on the stability of vitamin C, it is essential to show the effect of oxygen on the stability of this vitamin when its solutions are kept in test-tubes plugged with cotton. When orange juice is drawn under aseptic conditions into sterile test-tubes and plugged with cotton plugs, vitamin C is noticeably reduced in the course of about 2 weeks. Chart I gives the growth curves of guinea pigs fed sterile orange juice on the same day the orange juice was drawn; 7 days later; and 14 days later.

In all of this work guinea pigs of weights of from 200 to 250 gm. were placed on our scorbutic ration which consisted of 69 parts rolled oats, 25 parts alfalfa meal (steamed in the autoclave for 30 minutes at 15 pounds pressure), 5 parts casein, and 1 part common salt. On this ration most guinea pigs will show signs of scurvy, such as tender and swollen joints and difficulties in locomotion in 2 to 3 weeks. The criterion for the presence of vitamin C was the recovery type of experiment. When the animal showed signs of scurvy accompanied by loss in weight administration of the experimental material was then begun.

It is at once evident that when exposed to air through cotton plugs, feeding of the orange juice must begin on the same day it is drawn from the orange in order to cure guinea pigs of scurvy. On the other hand, if the orange juice is covered with *vaseline* as soon as drawn in order to keep the orange juice under strictly anaerobic conditions, then there appears to be little or no destruction of vitamin C when feeding is begun 18 days after the orange juice is drawn, as seen in Chart I. Animal 417 was cured of scurvy but died later of some cause other than scurvy. These results have been abundantly confirmed with tomato juice, as will be shown later.

The development of the technique of exclusion of the air through the use of a vaseline plug was of distinct advantage in studying this problem. The vaseline plug was from $\frac{1}{2}$ to $\frac{3}{4}$ inches in thickness and while it would be pushed up in the tube under certain conditions of gas production, yet it could be readily brought again into contact with the fluid of the test-tube by carefully melting.

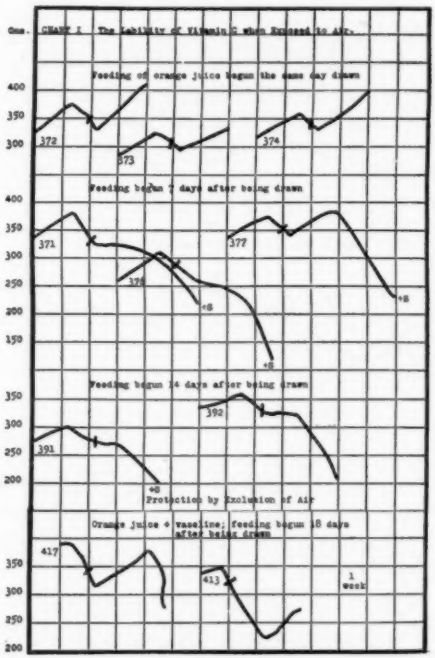


CHART I.

The direct effect on the stability of vitamin C of oxygen removal by a fermentation is beautifully illustrated in Chart II where orange juice was fermented with *yeast* and ordinary cotton plugs, but no vaseline used. This yeast fermentation of orange juice shows at once that there has been no destructive effect on vitamin C—a result in harmony with the observations of Zilva (4). On

the contrary, the activity of vitamin C persisted for a very long time—being retained for 51 days. This preservative effect was due to the liberal amount of carbon dioxide produced which removed most of the oxygen from the juice in a physical manner; but also, in addition, to the direct utilization of the oxygen in the juice by the growing organism. After 126 days the orange juice was found inactive, due, very probably, to a termination in the

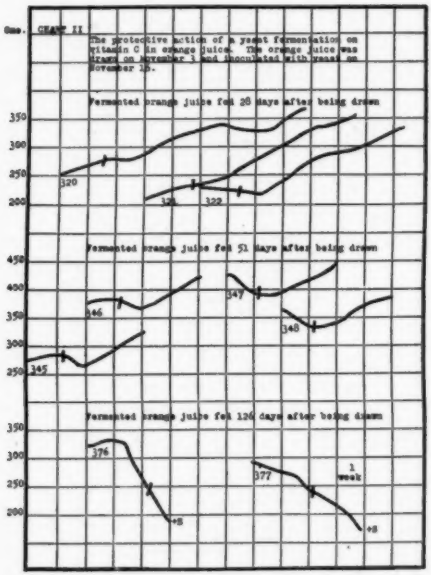


CHART II.

carbon dioxide production and subsequent absorption of oxygen from the atmosphere.

In this experiment the orange juice was drawn into test-tubes under aseptic conditions, covered with vaseline, and plugged with cotton. These tubes were allowed to stand 13 days and those that showed contamination were discarded. The remaining sterile tubes were then inoculated with a pure culture of bread yeast, after removal of the vaseline plug.

With a realization of the lability of vitamin C in sterile juices

when exposed to the air and the great protective value of anaerobic conditions brought about by the use of vaseline or by a yeast fermentation, a reliable method for the investigation of the stability of vitamin C to various organisms was available. For these studies canned tomato juice especially prepared by Kemp Brothers of Kokomo, Indiana, was used. The seeds and pulp had been strained out and the juice canned in tins. To this firm we are greatly indebted for this aid.

The experimental procedure was as follows: Canned tomato juice was pipetted under aseptic conditions into sterile test-tubes, inoculated with the organism to be investigated, calcium carbonate added when the acidity of the tomato juice was too great for the organism under investigation to grow, and the juice immediately covered with the vaseline plug. Gas formers will produce enough gas to push up the vaseline plugs and in consequence these plugs must be melted down frequently during the active fermentation period.

The organisms used were those whose biological behavior had already been studied. Organism 118-8 was isolated from sheep's manure by Fred, Peterson, and Davenport (5). It ferments xylose with the formation largely of lactic and acetic acids. It also ferments fructose, glucose, lactose, sucrose, and mannitol. It is active over a considerable range of acidity—of a pH varying from 3.0 to 8.6. The optimum temperature of growth is 30–35°C. This organism occurs in silage.

The organism *Streptococcus lactis* is so well known that its characteristics do not need to be described fully. It ferments glucose readily at room temperature. It also ferments lactose. It does not ferment pentoses, while 118-8 ferments pentoses as well as hexoses and disaccharides. These two organisms were considered especially valuable for study as to their effect on the stability of vitamin C. In addition the organism S-6, isolated from cheese, was studied. This organism was of a proteolytic type.

The following experiments were planned and the treated tomato juice allowed to ferment at room temperature and to stand for *24 days* before feeding; the fermented juice was fed at a level of 4 cc. per individual daily.

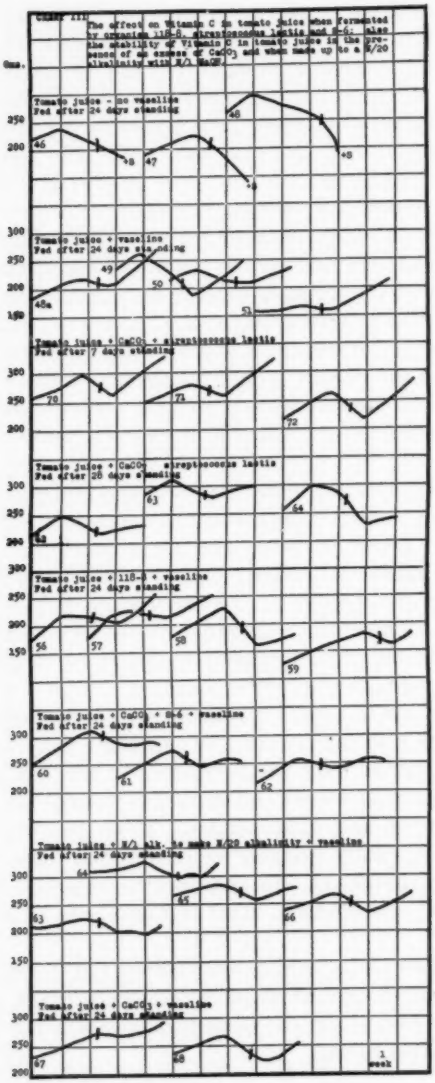
Chart III.
The effect on Vitamin C in tomato juice when fermented by organism 118-8, streptococcus lactis and C-6, also the stability of Vitamin C in tomato juice in the presence of an excess of $CaCO_3$ and when made up to a N/20 alkalinity with N/1 NaOH.
Cmg.
Tomato juice - no vaseline
Fed after 24 days standing
Tomato juice + vaseline
Fed after 24 days standing
Tomato juice + $CaCO_3$ + streptococcus lactis
Fed after 7 days standing
Tomato juice + $CaCO_3$ streptococcus lactis
Fed after 20 days standing
Tomato juice + 118-8 + vaseline
Fed after 24 days standing
Tomato juice + $CaCO_3$ + C-6 + vaseline
Fed after 24 days standing
Tomato juice + N/1 alk. to make N/20 alkalinity + vaseline
Fed after 24 days standing
Tomato juice + $CaCO_3$ + vaseline
Fed after 24 days standing
1 week

Chart III.

1. Sterile tomato juice exposed to air that circulated through the cotton plugs.
2. Sterile tomato juice, plus vaseline.
3. Sterile tomato juice, plus vaseline, plus calcium carbonate.
4. Sterile tomato juice, plus vaseline, plus calcium carbonate, plus *Streptococcus lactis*.
5. Sterile tomato juice, plus vaseline, plus calcium carbonate, plus S-6.
6. Sterile tomato juice, plus vaseline, plus 118–8.
7. Sterile tomato juice, plus vaseline, plus normal NaOH solution to make N/20 alkalinity.

No calcium carbonate was added in Experiment 6 where organism 118–8 was used because this organism grew very well in the tomato juice without reduction of its acidity. As seen in Chart III tomato juice without vaseline failed to cure scorbutic guinea pigs, thus demonstrating again the destruction of vitamin C in sterile tomato juice when exposed to air in cotton-plugged test-tubes. Tomato juice plus vaseline with or without calcium carbonate cured scorbutic guinea pigs. Tomato juice fermented with organism 118–8 and S–6 under the conditions described effected a cure, indicating that these organisms do not destroy vitamin C, within the limits of the experiment.

The tomato juice fermented with *Streptococcus lactis*, though not effecting a cure, greatly prolonged the lives of the animals, thus indicating partial destruction of vitamin C when fed after 28 days of fermentation with this organism. On the other hand, when the tomato juice was fermented with this organism for but *7 days* and then fed to the scorbutic guinea pigs, a cure of scurvy resulted. From these experiments it is difficult to decide whether this organism destroys vitamin C. It certainly does it slowly, if at all, and it is possible that the long time fermentation (28 days) may have allowed some access to oxygen. This hardly seems the case because the potency of the fermented mixture inoculated with S-6 and 118–8 was very distinct even after 24 days.

The destructive effect of an alkaline medium on vitamin C is well known. Orange juice made to a N/20 alkalinity loses practically all of its vitamin content in 24 hours (6). From the results secured (Chart III) it is evident that such alkaline tomato juice retains a considerable portion of its original vitamin C content even after *24 days* when kept under strictly anaerobic conditions made possible by the use of vaseline. While the guinea pigs were

not completely cured of scurvy, their lives were very much prolonged.

There is no logical reason why microorganisms should not be found that will destroy vitamin C, but it is altogether probable that its disappearance from fermented corn (silage) and fermented cabbage (sauerkraut) is to be referred to a destruction through contact with oxygen, which is low in amount but seldom completely absent in the gases taken from the silage mass (7). This is especially true unless the silage is taken far from the surface or periphery of the silo. So far as we are aware the gases in a mass of fermented cabbage have not been investigated.

SUMMARY.

1. Attempts to determine the cause for the disappearance of vitamin C from fermented corn (silage) and fermented cabbage (sauerkraut) have been made. The action of several organisms occurring in silage have been studied, but it has not been shown that they destroy vitamin C.

2. For this study a technique involving the use of vaseline for exclusion of air from the fermenting mixture has been developed. With this technique, the destructive action of oxygen on vitamin C can be excluded.

3. While all of the organisms occurring in silage have not been included in these studies, yet it is very probable that the destruction of vitamin C in masses of fermenting plant tissue is to be referred to the oxygen still retained in the mass.

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# SOME OBSERVATIONS UPON THE RÔLE OF CYSTINE AND CERTAIN MINERAL ELEMENTS IN NUTRITION.*

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(Received for publication, August 20, 1925.)

In connection with studies upon the determination of cystine by means of feeding experiments, recently reported from this laboratory (1) there was observed a strikingly good appearance of tissues at autopsy in young animals stunted by feeding upon diet good in other respects but deficient in cystine, as compared with the condition of animals correspondingly stunted by vitamin deficiencies.

Moreover, animals after relatively long stunting by shortage of cystine (8 weeks in young albino rats, or nearly the entire period between weaning and maturity) were able, upon being returned to a normal diet, to resume growth at a rate fully normal for their size and to make normal records in reproduction and the rearing of young.

Inasmuch as the basal diet used in these cystine-stunting experiments consisted essentially of whole milk overdiluted with starch together with small amounts of supplements to ensure the adequacy of the mineral and vitamin factors, it becomes of interest to correlate these observations with the results of experiments which have been made in this laboratory upon animals fed diets consisting exclusively of milk or in which milk was the chief constituent.

On milk alone rats thrive up to an age and development corresponding to those of children 10 to 15 years old. On a diet of whole milk powder without any supplements whatever young rats, both males and females, in this laboratory have grown to

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average weights (142 gm. for males and 129 gm. for females) at 80 days well within normal limits and above the averages attained by rats of the same strain on our Laboratory Diet A (2), which as judged by all current standards has been shown to be adequate both for growth and reproduction. On a diet of whole milk powder plus 1 per cent table salt young males with average weights of 190 gm. at 90 days are above the Donaldson standard and the females with average weights of 138 gm. at the same age are within the limits set for that standard. On this diet fair reproduction has been obtained with proven fertility in the second generation. One female bore young at the age of 88 days and another at 96 days. The animals on these diets were not kept for longevity records but two were 455 days old when killed.

For longer feeding experiments whole wheat, in the experience of this laboratory, has high value as a supplement for whole milk.

A family of rats upon a diet consisting of five-sixths whole milk powder, one-sixth ground whole wheat, and table salt in the proportion of 2 per cent of the wheat has made a good record for growth, maximum adult size, reproduction, and longevity. The male gained 99 gm. during the first 28 days after weaning, reached a maximum weight of 369 gm., and lived to be 812 days old. The 2 females gained 96 and 73 gm., respectively, in the first 28 days after weaning and bore a total of 111 young, 46 of which were reared with an average weight of 44 gm. at 28 days of age. 1 female gave birth to a litter of 2 young at the unusually advanced age of 682 days. These were not reared, however. The females lived to be 823 and 956 days old, respectively.

A family in direct comparison with the one just referred to but upon a diet consisting of two-thirds whole milk powder, one-third ground whole wheat, and table salt in the proportion of 2 per cent of the wheat (Diet D) made a record which is typical of many others upon the same diet. The male gained 114 gm. in the first 28 days after weaning, reached a maximum weight of 419 gm., and lived to be 887 days old. The females gained 89 and 86 gm. in the first 28 days after weaning and lived 772 and 874 days, respectively. A total of 91 young were born of these 2 females of which 35 were reared with an average weight of 50.9 gm. at 28 days of age. 1 of these females showed an unusual length of reproductive life for her first litter was born when she was 80 days

of age, and her last when she was 618 days. Her last litter was reared.

Complete life records of 16 animals in generations from the second to the sixth on the diet containing two-thirds whole milk powder have been kept and are summarized briefly, together with the record of one lot of the sixth generation which was killed when the rats were 554 days old. The data which follow are the averages of 20 rats—5 males and 15 females. There was no conscious selection of the animals kept for the life histories and doubtless other families would have made equally good records. Of this group the earliest death was at 427 days and the latest at 1076; 8 animals (nearly half of the group) lived to be more than 840 days of age which would correspond to 70 years in human life. All of the females reared young. 755 young were seen or an average of 50 young per female. 522 young were reared, or 34.8 young per female, that is 69.1 per cent.

The animals in our laboratory all live upon bare wire netting through their entire lifetime and the young are reared without bedding except for a small amount of pure cellulose paper cut in small pieces and given after the young are born but omitted after they are 2 weeks old. When these rather harsh conditions of living are considered the records made by the animals on this diet both for longevity and for rearing of young seem extremely good.

The very high supplementing value of whole wheat for whole milk as indicated by the experiments just described does not seem to be entirely explainable on the ground of roughage, or of dilution with carbohydrate, for workers in other laboratories do not report equally good results on diets consisting chiefly of milk but containing agar-agar and starch, or dextrin. Mattill and Conklin (3) reported normal growth on a diet composed of 55 per cent dry whole milk, 40 per cent starch, and 5 per cent butter fat but none of the young born were reared. Mattill and Stone (4) reported that reproduction was not successful on any of their diets consisting of whole milk powder (50 per cent or more) diluted with starch and lard, either with or without the addition of McCollum's salt mixture No. 185 or iron citrate. In their experience yeast addition to a 60 per cent milk powder ration improved it in respect to reproduction. In 1924 a report from the same laboratory (5) de-

scribed changes in the reproductive system of the rats kept upon a diet composed largely of whole milk powder, which resulted in sterility. They found that 5 to 10 per cent wheat embryo would restore fertility to the females and would prevent the degenerative process in the males. These investigators ascribed their dietary deficiency to the lack of the fat-soluble substance which Evans and Bishop (6) have found essential for normal reproduction.

Sure (7) reported that none of the young born of females on his milk protein diets which he believed were adequate in the mineral and vitamin factors were reared and that neither purified protein nor amino acid supplements corrected the deficiency but that certain natural foods were efficient supplements for successful lactation.

However, Osborne and Mendel (8) have had normal growth, reproduction, and normal growth in the second generation upon a diet composed of whole milk powder 60 per cent, starch 12 per cent, lard 28 per cent; and Anderegg (9) has published results of his studies with milk and milk products as the sole source of protein and of vitamins in the diet which show that his rats were not sterile upon these diets although he says he found high mortality among the young. Daniels and Stearns (10) have recently reported that 5 to 10 gm. of cooked soy bean powder per liter of boiled milk renders the milk diet adequate for successful reproduction and lactation. They found that soy bean ash was also effective as a supplement for the milk diet as evidenced by the rearing of three successive generations on milk plus soy bean ash. They suggest that the improvement which they found with the soy beans was due to small amounts of certain mineral elements which the soy beans supplied but which are not usually taken into account in dietary planning. Their success with an artificial salt mixture, containing aluminium, silicon, manganese, fluorine, iron, and iodine, as a supplement for whole milk is also reported.

It seems probable that the high value of whole wheat as a supplement for whole milk powder may be due, largely at least, to its ability to supplement or to conserve the mineral factors.

In a consideration of the possible supplements of the mineral factor of whole milk attention is naturally given first to iron. In our experience, the addition of ferric citrate to the whole milk powder had little effect, except to give added color to the ears

and eyes of the animals while they were young. Adult rats which had had added iron from weaning did not show increased body weight nor increased iron content over those which had had only the iron of the whole milk powder. The iron of milk although small in amount is able to go a surprisingly long way in nutrition.

In general our results with diets containing two-thirds, or more, whole milk powder have been excellent. With exclusive milk powder diets we have some evidence of the same nutritional performance which other investigators have reported but find whole wheat an exceptionally good supplement for both growth and successful reproduction. Since the failure in our experience in the exclusive milk diets was more marked in the suckling of the young than in the production of normal young it appears that this diet must supply a sufficient amount of vitamin E as Evans and Burr (11) have shown it should; and the failure must, therefore, be sought in some other factor. It seems probable that there may be a relative shortage of the less prominent mineral elements which may be necessary for normal gland secretion, or that the proper mineral balance for membrane equilibrium cannot be maintained with maximum ease and efficiency. The fact that the cystine-stunted animals, which had had the benefit of the less prominent mineral elements contained in the Osborne and Mendel salt mixture of the cystine-limited diet, recovered with noticeably successful suckling of their young gives added weight to this view of the mineral factors of the diet.

The author is greatly indebted to Professor H. C. Sherman for his constant interest and many helpful suggestions during the course of this work.

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# CONDENSATION PRODUCTS OF ACETOACETIC ESTER.

## II. OXIDATION AND POSSIBLE RELATIONSHIP TO ANTIKETOGENESIS IN THE ANIMAL BODY.*

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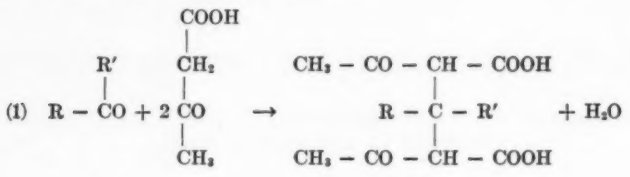
(Received for publication, August 1, 1925.)

According to the theory developed by Geelmuyden (1), Ringer (2), Woodyatt (3), and Shaffer (4), glucose is antiketogenic in human metabolism because it or one of its decomposition products combines with acetoacetic acid or other ketogenic molecule forming a substance which is more easily oxidized in the body than are the unchanged acetone bodies. Shaffer (5) observed the rapid oxidation of acetoacetic acid by hydrogen peroxide in alkaline solution when glucose is present. Shaffer and Friedemann (6) have studied the "ketolytic reaction" in some detail and have demonstrated that glycol aldehyde, glyceric aldehyde, etc., possible decomposition products of glucose in the animal body, also readily bring about the oxidation of acetoacetic acid by peroxide in alkaline solution.

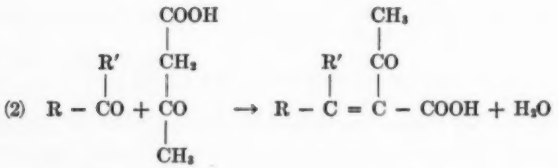
Shaffer (7) considers that the reaction by which these compounds promote the *in vitro* oxidation of acetoacetic acid is probably analogous to the antiketogenic action of glucose or its decomposition products in the animal body, and that the reaction is the formation of condensation products with acetoacetic acid which are more easily oxidized by the peroxide than the unchanged acid. According to Shaffer (8) the condensation of acetoacetic acid with the antiketogenic molecule *in vitro* and *in vivo* probably proceeds as a Knoevenagel type (9) of reaction in which an aldehyde or ketone condenses with a 1, 3-diketone

* The first paper of this series appeared in *J. Am. Chem. Soc.*, 1925, xlvii, 2780.

or ketonic ester. The reaction is supposed to take place according to one of the following equations:



I. II.



III. IV.

in which formulas I and III represent the antiketogenic molecule and formulas II and IV the condensation product which is more easily oxidized than acetoacetic acid. This interpretation is rendered more plausible by the work of Dakin (10) who has shown that condensations of this type may be catalyzed by certain amino acids and peptones, that is, under conditions which may exist in the animal body.

Many compounds of acetoacetic *ester* of type II are known, but so far as the writer is aware only one of the free acids, namely vanillylidene-bis-acetoacetic acid (11). Attempts to prepare condensation products of the acid by hydrolysis of the ester product generally result in the loss of water with ring closure and formation of cyclohexenone derivatives (12). Direct preparation is difficult because of the instability of acetoacetic acid.

A search of the literature has revealed no compound of acetoacetic acid of the type represented by formula IV. Similar compounds of the ester, upon hydrolysis, readily break at the double bond and reform the constituents, so that preparation by this procedure has not been successful (13). Analogous compounds of malonic acid, however, are known (14).

If it is true that condensation products of acetoacetic acid are oxidized in the animal body more readily than acetoacetic acid itself, it occurred to the writer that these compounds should

also be more easily oxidized *in vitro* than acetoacetic acid. This is presumably the case in the experiments of Shaffer and Friedemann, although the hypothetical condensation products were not isolated. Accordingly, a study was undertaken of the reducting properties (ease of oxidation) of various condensation products of acetoacetic acid in comparison with those of acetoacetic acid.

Because of the present impossibility of obtaining a number of condensation products of the free acid the corresponding ester derivatives were employed. When these compounds are boiled with appreciably alkaline solutions, as was the case in most of the tests, it is logical to assume that a certain amount of the transitory free acetoacetic acid condensation product exists. The reducing action of the compounds toward Tollens' reagent, alkaline copper solutions, and alkaline methylene blue was used as a basis of comparison.[1]

It has been found in confirmation of the hypothesis, that many of the condensation products of acetoacetic ester (acid) are far more easily oxidized than acetoacetic ester (acid) itself. In fact, some of them rank among the most powerful reducing compounds known. It is an interesting fact that, despite the great amount of work which has been done upon the condensation products of acetoacetic ester, apparently no investigator has previously noted the reducing properties of these compounds.

Commercial acetoacetic ester shows considerable reducing power toward Tollens' reagent, slight toward hot alkaline copper solutions, and very little toward hot alkaline methylene blue. When dissolved in ether and washed repeatedly with saturated sodium chloride solution and water until the washings showed practically no reducing action on Tollens' reagent, and the ether evaporated, a product was obtained which acted upon Tollens' reagent slowly, had no action upon hot alkaline copper solutions, and practically no effect upon hot alkaline methylene blue. When this purified material was distilled in vacuum (0.7 to 0.8 mm.) the first fraction possessed reducing properties of the same order as the undistilled compound, but the undistilled ester, remaining in the distilling flask, had acquired stronger reducing properties.[2]

[1] Potentiometric studies are planned for the future.

[2] It may be that a trace of acetone, or some other decomposition product of acetoacetic ester, condenses with the ester to form an easily oxidized compound during the procedure of distillation.

In contrast to the very slight reducing action (if any) of acetoacetic ester (acid) toward these reagents is the pronounced action of methylene-bis-acetoacetic ester, ethylidene-bis-acetoacetic ester, benzylidene-bis-acetoacetic ester, vanillylidene-bis-acetoacetic ester, $\alpha'$-acetoacetic ester-$\alpha$-methyl-$\beta$-furan carboxyllic acid ethyl ester, and a condensation product of glucose and acetoacetic ester which has been prepared in this laboratory. Benzylidene malonic ester (acid), while not a strong reducing agent, is more powerful than malonic ester (acid).

## EXPERIMENTAL PART.

The compounds used were carefully prepared and purified. The procedures used in the preparation of methylene-, ethylidene-, benzylidene-, and vanillylidene-bis-acetoacetic ester condensation products and benzylidene-malonic ester were essentially those developed by Knoevenagel and coworkers (15). $\alpha'$-Acetoacetic ester-$\alpha$-methyl-$\beta$-furan carboxyllic acid ethyl ester was prepared as outlined in the first paper of this series (16).

### *I. Methylene-Bis-Acetoacetic Ester.*

*Tollens' Reagent.*—When ammoniacal silver nitrate was added to a thoroughly shaken suspension of the compound in dilute alkali, reduction took place instantly.

*Fehling's Solution.*—A small amount of reduction occurred when the compound was boiled with the reagent. If, however, the Fehling's solution was diluted four to five times reduction readily occurred. Boiling the compound with 10 per cent alkali destroys its reducing properties.[3] In this is found the explanation of the slight action upon undiluted Fehling's solution.

*Benedict's Solution.*—The compound is very insoluble in the reagent and when boiled with it floats around as droplets with very little reducing action. When the compound was first suspended in dilute alkali, thoroughly shaken, the reagent added, and the mixture boiled, there was rapid reduction.

*Alkaline Methylene Blue.*—The boiling solution was quickly decolorized.

### *II. Ethylidene-Bis-Acetoacetic Ester (M.P. 79–80°C.).*

The action of this compound toward Tollens' reagent, alkaline copper solutions, and alkaline methylene blue was entirely similar to that of methylene-bis-acetoacetic ester. In this case, also, boiling the compound with dilute alkali destroys its reducing properties toward Fehling's solution.

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[3] The loss of reducing power is probably due to conversion of the compound into a cyclohexenone derivative (17).

### *III. Benzylidene-Bis-Acetoacetic Ester (M.P. 152–153.5°).*

*Tollens' Reagent.*—The compound is quite insoluble in the reagent and reduction was slow. If, however, the compound was dissolved in 3 to 4 cc. of alcohol, 1 cc. of Tollens' ammoniacal silver nitrate added, and then 4 to 5 drops of 10 per cent sodium hydroxide, reduction took place readily. The proportions specified must be rather closely adhered to else a precipitate of silver oxide may be obtained.

*Fehling's Solution.*—A deep blue solution containing suspended cuprous oxide was formed when the compound was boiled with the reagent. Upon standing, the oxide settled out in considerable amounts. Diluted Fehling's solution was reduced much more readily. Boiling with dilute alkali destroys the reducing properties of the compound.

*Benedict's Solution.*—No action occurred with the unaltered reagent, the crystals remaining unmolten and undissolved when boiled. When to the boiling solution an equal volume of dilute alkali was added, and heating continued, the reagent was readily reduced.

*Alkaline Methylene Blue.*—The boiling solution was quickly decolorized.

### *IV. Vanillylidene-Bis-Acetoacetic Ester (M.P. 164–165°).*

*Tollens' Reagent.*—The action was quite similar to that of benzylidene-bis-acetoacetic ester.

*Fehling's Solution.*—The undiluted reagent was reduced very slowly. When boiled with the solution which had been diluted four to five times rather rapid reduction occurred despite the fact that a large proportion of the compound remained in the reagent as undissolved crystals. The reducing power of the compound is destroyed by boiling with dilute alkali.

*Benedict's Solution.*—When an alcoholic solution of the compound was added to a boiling mixture of Benedict's solution and dilute alkali reduction readily took place.

*Alkaline Methylene Blue.*—The compound decolorized the hot solution quickly.

### *V. α′-Acetoacetic Ester-α-Methyl-β-Furan Carboxyllic Acid Ethyl Ester.*

*Tollens' Reagent.*—Instant reduction when a finely divided suspension of the compound in 10 per cent alkali was treated with ammoniacal silver nitrate.

*Fehling's Solution.*—Powerful reduction of the boiling solution.

*Benedict's Solution.*—Very strong reduction of the boiling solution.

*Alkaline Methylene Blue.*—Decolorized instantly *in the cold.*

### *VI. Condensation Product of Glucose and Acetoacetic Ester.*

The compound reduced Fehling's solution within a few seconds *in the cold,* and decolorized exceedingly weak alkaline methylene blue *in the cold.* Tollens' reagent was reduced instantly.

*VII. Benzylidene-Malonic Ester (B.P. 140–145° at 0.7 to 0.8 Mm.).*

The compound was separated as thoroughly as possible from benzaldehyde by steam distillation and fractional distillation in vacuum (0.7 to 0.8 mm.). The purified material possessed no odor of benzaldehyde when warm, and tests with Schiff's reagent revealed only a small trace of aldehyde after many hours standing.

*Tollens' Reagent.*—The compound is extremely insoluble in the reagent and reduction was slow. When, however, it was tested in alcoholic solution, as in the case of benzylidene-bis-acetoacetic ester, reduction took place quickly. Tests using a small amount of benzaldehyde showed that reduction by the purified compound was not due to the trace of aldehyde which was present as an impurity.

The compound does not reduce alkaline copper solutions nor decolorize hot alkaline methylene blue.

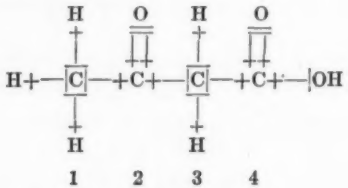
*VIII. Malonic Ester (Kahlbaum Product).*

*Tollens' Reagent.*—No reduction even though a large amount of heat was generated upon mixing with the reagent.

The compound does not reduce alkaline copper solutions, nor does it decolorize hot alkaline methylene blue.

DISCUSSION.

Hanke and Koessler (18) have pointed out that the chemical properties and relationships of acetoacetic acid are in accord with the following electronic configuration:

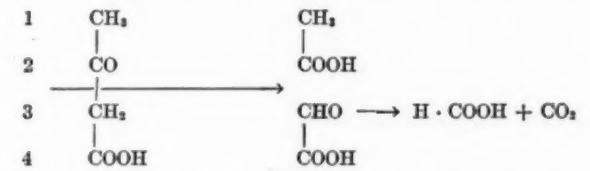



V.

in which there is alternating polarity of the carbon atoms. Carbon atoms 2 and 4 should be more polar than 1 and 3 due to combination with oxygen and consequently greater electron displacement. The unions of carbon atoms 1 and 3 to hydrogen are presumably only weakly polar.

According to this conception carbon atoms 1 and 3 are the only ones capable of being oxidized, that is losing electrons. Of

these carbon atoms, 3 should lose electrons or be oxidized most readily. This is true because it is adjacent to two strongly positive carbon atoms, 2 and 4, which attract its electrons and cause them to be held less strongly, with the result that they are lost more readily to an oxidizing agent. Enolization, with each shift of the hydrogen atom, also exposes the electrons of carbon 3 and renders it more easily oxidized than carbon 1. The facts are that in cases of mild oxidation of acetoacetic acid only carbon atom 3 is oxidized, carbon 1 appearing in a molecule of acetic acid with carbon 2. Thus, acetoacetic acid, when oxidized with hydrogen peroxide in alkaline solution, gives, according to Dakin (19) acetic, glyoxylic, and formic acids, and carbon dioxide. Dakin formulates the action as follows:




Under the conditions of the "keytolytic" experiments described by Shaffer and Friedemann[4] the products of its oxidation are 1 mol of acetic acid and 2 of carbon dioxide. Very evidently only carbon 3 has been oxidized. Simply an addition of OH to positive carbon 2 has occurred, which involves no oxidation, and carbon 4 remains unchanged.

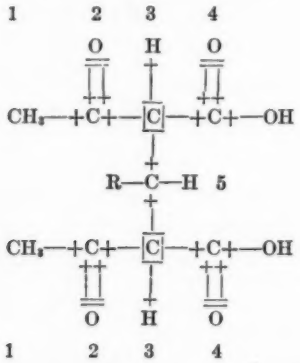
Theory, then, leads to the conclusion that in the oxidation of acetoacetic acid initial oxidation should take place at carbon atom 3 because of the greater ease with which electrons should be lost at this atom. The facts substantiate the theory.

The experimental work reported above shows conclusively that the introduction of methylene, ethylidene, benzylidene, vanillylidene, etc., radicals into the acetoacetic acid molecule in the place of hydrogen attached to carbon atom 3 has greatly increased the ease of oxidation of the molecule. It is barely conceivable that the group substituted for hydrogen can serve as the initial point of oxidation in the condensation products, because most of these are hydrocarbon or other radicals which show marked resistance to oxidation. We must conclude, therefore, that carbon atom 3 of the acetoacetic acid molecule con-

[4] Shaffer, P. A., and Friedemann, T. E. (8), p. 611.

stitutes the position of greatest ease of oxidation in the "condensation products of acetoacetic acid" as well as in the acid itself. Now since these condensation products are much more easily oxidized than acetoacetic acid, it must follow that the substitution of these various groups for hydrogen has greatly increased the ease with which carbon atom 3 loses electrons. Such a conclusion is entirely in accord with the electronic configuration of the condensation products which will now be considered.

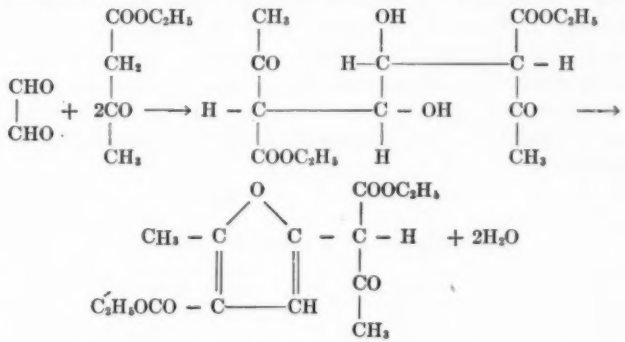
The methylene, ethylidene, benzylidene, and vanillylidene condensation products of acetoacetic acid (ester), and others of this type, have the following electronic configuration around carbon atom 3 of the acetoacetic acid chain:



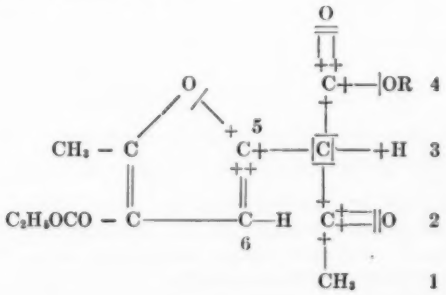

in which carbon 3, the possessor of a full quota of electrons, is surrounded by three positively charged carbon atoms. Carbon 5 was derived from a carbonyl group and, barring subsequent electronic shifts, possesses at least two positive charges, and it may be three, especially in the case of compounds in which R is aryl (20). The essential change in the electronic relationships of carbon atom 3 in these condensation products from those in acetoacetic acid is, then, that a "weakly polar hydrogen" attached to carbon 3 in the acid has been replaced by a "more strongly positive" carbon atom in the condensation product. Consequently, the electrons of carbon atom 3, which are now subjected to the attraction of *three* adjacent, strongly positive

fields of force, are held less firmly than in the case of acetoacetic acid, and the compound is more easily oxidized. In all of these condensation products there is still the possibility of enolization due to the shift of hydrogen from carbon 3 to the oxygen of carbon 2. Just as in the case of acetoacetic acid, this facilitates the oxidation of the condensation product as a result of exposure of electrons on carbon 3.

A similar interpretation applies to the ease of oxidation of the other condensation products of acetoacetic acid in which a hydrogen of carbon 3 is replaced by a carbon atom with a stronger electropositive field of force. In the case of $\alpha'$-acetoacetic ester-$\alpha$-methyl-$\beta$-furan carboxyllic acid ester, which is formed from glyoxal and acetoacetic ester according to the equations (21)



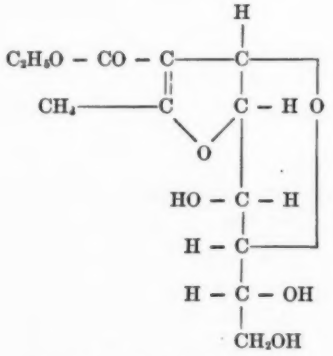
the essential electronic relationships of carbon atom 3 of the acetoacetic acid chain are



Carbon atom 5 should have four positive valence forces. The union to oxygen is positive, and that to carbon 3 is also positive if there has been no electronic shift (intramolecular oxidation reduction) after condensation.

When hydrogen of carbon 3 shifts to oxygen of carbon 2 in the process of enolization a double bond is formed between carbon atoms 2 and 3. This, with the double bond between carbon atoms 5 and 6, constitutes a conjugated double bond system. Since the double bond is negative at carbon 3 it follows that the double bond unions at carbon 5 are positive, and carbon 5 has four positive valence forces. Here we have a case in which carbon 3 of the acetoacetic acid chain is adjacent to three "wholly" positive carbon atoms, a condition which should cause the electrons of carbon 3 to be lost very readily and the compound to be easily oxidized. As a matter of fact it is the most easily oxidized of the compounds thus far discussed, being the only one which decolorizes alkaline methylene blue in the cold.

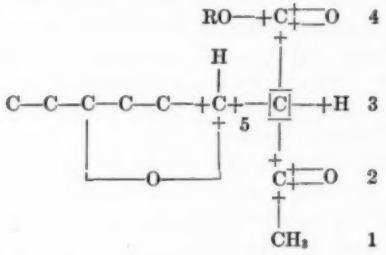
Glucose condenses with acetoacetic ester in the presence of zinc chloride[5] to form a compound which presumably has the formula:



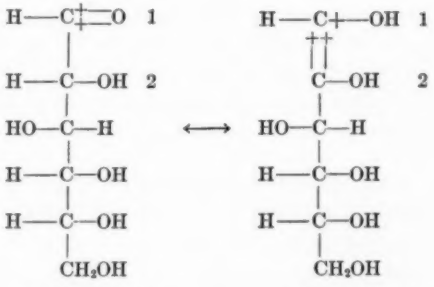

When this compound is dissolved in concentrated hydrochloric acid, and the solution neutralized, a heavy oil is obtained in

[5] The writer has so far been unable to find a reaction of this type in the literature. It seems to be a new reaction of glucose and possibly of the sugars in general. Attempts are now being made in this laboratory to extend the reaction to other sugars and also other 1,3-diketones and ketonic esters. A preliminary report will appear in the near future.

which, apparently, the furan ring involving the acetoacetic ester molecule is opened. At the same time water is eliminated at some position in the carbohydrate chain, presumably in the furan ring, though the exact position has not yet been determined. The electronic configuration around carbon 3 in the acetoacetic acid chain of this second compound is:



Carbon atom 5 of the compound posesses three positive valence forces. In the glucose molecule, acting as an aldehyde, the aldehyde carbon is attached by two valences to negative oxygen. These valence forces are consequently positive. When this aldehyde form passes into the



Aldehyde form. 1,2-dienol form.

1,2-dienol form, one positive valence of carbon attached to the oxygen of the carbonyl group becomes one of the valence forces of the double bond in the dienol by which carbon 1 is attached to carbon 2, and is consequently positive. Now Stieglitz (22) has pointed out that both the valence forces of a carbon atom which are concerned in a double bond tend to have the same

charge, and since in these transformations of glucose from one form to another presumably no intramolecular oxidation reduction occurs, carbon atom 5 of the condensation product of glucose should have three positive charges as shown in the formula. Here again carbon 3 of the acetoacetic acid chain is adjacent to three strongly positive fields of force and should lose electrons readily and be easily oxidized. As pointed out in the experimental work the compound is remarkable in that it reduces Fehling's solution quickly (within a few seconds) *in the cold* and decolorizes weakly alkaline solutions of methylene blue *in the cold*. It is accordingly a more powerful reducing agent than either of its components.

It is to be expected that different groups substituted for hydrogen of carbon 3 in acetoacetic acid should produce compounds of varying reducing powers due to the varying degrees of polarity of the positive carbon atoms introduced. Thus, a highly polar carbon atom of three positive charges might produce a compound of greater reducing power than one of four positive, but less polar, charges. The compounds of glyoxal and acetoacetic ester and glucose and acetoacetic ester seem to represent a case of this kind.

The increase in the reducing power of malonic ester (acid), due to the introduction of the benzylidene radical in the place of hydrogen, may also be explained according to the theory that the increase in the intensity of the positive fields of force on the atoms adjacent to the electron-bearing carbon atom renders these electrons more easily lost and the compound more readily oxidized.

The study of the oxidation of condensation products of acetoacetic ester (acid) quantitatively *in vitro* and in the animal body will shortly be undertaken.

I wish to take this opportunity to thank Professor P. A. Shaffer for suggestions and criticisms during the course of this investigation.

## SUMMARY AND CONCLUSIONS.

1. It has been shown by *in vitro* experiments that certain condensation products of acetoacetic ester (acid) have higher reducing potentials, or are more easily oxidized, than aceto-

acetic ester (acid). This is in accord with the theory developed by Shaffer and others as to the mechanism by which glucose prevents ketosis in human metabolism, and also in accord with Shaffer's interpretation of the *in vitro* antiketogenic action of glucose, its decomposition products, etc.

2. An electronic interpretation has been suggested which accounts for the greater ease of oxidation of *condensation products* of acetoacetic ester (acid) and which demonstrates the soundness of the chemical basis for the current theory concerning antiketogenesis.

3. What is believed to be a new reaction of glucose, and possibly the sugars in general, is reported.

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# BODY FLUIDS OF THE HONEY BEE LARVA.

## II. CHEMICAL CONSTITUENTS OF THE BLOOD, AND THEIR OSMOTIC EFFECTS.

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(Received for publication, July 18, 1925.)

Certain peculiarities noted in a study of the respiratory exchange and pH of the blood of the bee larva (Bishop, 1923, *b*) prompted a study of the chemical constituents of this fluid.[1] A comparison of its characteristics and properties with the blood of mammals proves of considerable interest. The sugar metabolism, while high during the feeding stage, approaches during rest the values for a resting mammal. The salt concentrations on the other hand are so different from those of most animals as to indicate that the "sea water" analogy fails for this animal, though there is still probably a specific salt ion balance. Further, amino acids seem to play a peculiar rôle both in osmotic activity and in buffering the relatively large amounts of calcium and magnesium present.

In consideration of what follows, it may be of interest to note that the bee larva subsists on honey and plant pollen, gathered by the mature bees from a large variety of sources. The inorganic constituents of the honey, which are largely glucose, are probably

[1] Two papers by Muttkowski (1923, 1924) deal with the blood chemical constituents of various insect larvæ and adults, the results being qualitative, from microchemical determinations. Clotting is reported in these bloods, with presence of fibrin, also gelatin. No clotting has been observed in bee blood, in spite of very high calcium. Literature on microchemical qualitative results of previous workers is there reviewed.

relatively insignificant, compared to those of the pollen. The same can be said of proteins. The salt constituents are apparently conditioned by the plant source. The plant oils are not digested, though some are ingested, and voided with a mass of undigested pollen husks at the beginning of pupation. In early larval life (first 3 days) the animals are fed by the worker bees on a partially digested material regurgitated by the worker bees, roughly half protein and half glucose. The inorganics of this material have not been determined.

After the first 3 days the larvæ are fed on honey and pollen. The honey contains other sugars than glucose, several of which can be utilized at least by the mature bees. (E. F. Phillips, personal communication.) At 5½ days the larva stops eating, spins a cocoon and pupates, in the wax cell of the comb. By far the largest growth takes place in the last 2½ days (15 mg. at 3 days, 155 mg. at 5½ days). The larval blood was taken at 5 days or later; some of the samples were taken at the 6th or 7th day while the larvæ were sealed over and spinning.

After pupation, the larval cells undergo rapid metamorphic changes (Bishop, 1922, 1923, *a*) by which the larval "fat body" cell constituents are prepared for use by the growing tissues of the mature insect. These tissues are developed from embryonic fundaments in the larvæ, accompanying which most of the larval tissues are histolyzed. The blood from the "pupal" stage was taken shortly after spinning of the cocoon, in the so called prepupal stage, when the above histolytic process had not gone very far. The main changes to be expected from the larval conditions should appear in the sugar and in the protein fragments, rather than in the inorganic constituents. In later stages the body fluids are so contaminated with cell debris that results would be misleading.

The samples of blood consisted of lots of from 0.1 to 10 cc., depending on the tests to be made, obtained by pricking the dorsal blood space with a needle, and either allowing the carcasses to drain from the edge of a funnel, or else taking the blood up directly into a capillary pipette. About thirty drone or male larvæ were required per cc. of blood; or fifty to sixty worker larvæ. Many more pupæ were required, for there is a rapid reduction in blood volume with pupation.

*A. The Chemical Constituents of Bee Larval Blood.*

Table I[2] presents data from a number of determinations over a period of 2 years. The results do not show that differences in pollen collected at different seasons affect the blood constituents. On the average 85 per cent of the non-protein N is in the form of amino N. Other determinations, Table II,[3] indicate that the ratio between total N and non-protein N is about 2 to 1. The amino N is about fifty times the normal for mammalian plasma. The results show no significant differences in organic constituents for sex. The highest sugar results may be due to contamination of the blood. The larvæ when disturbed often regurgitate small amounts of stomach contents, and furthermore, there is often food, mainly honey, in the cell with them. Since the blood was sometimes obtained by allowing the fluid to drain from the larvæ, small amounts of glucose may have been admixed from the exterior of the animals. The lower values were obtained when the blood was taken up with a pipette from a prick in the side of the body; the doubtful values are in parentheses. The high sugar value which prevails during feeding, falls during the activity of spinning, being formed, doubtless, from glycogen reserves in the cells after depletion of the blood supply. (Straus, 1911, gives data for glycogen depletion during pupation.) The acidity of the blood increases with spinning (Bishop, 1923, *b*), and lactic acid is presumably produced. Lactic acid determinations made were uncertain, though high in general. Some substance was present that interfered with the reaction (Clausen, 1922).

Table I presents data on the inorganic constituents. The inorganic phosphorus is 1070 per cent of the value for mammalian blood, the calcium 150 per cent, magnesium 800 per cent, potassium

[2] The chemical determinations of Table I were carried out by A. P. Briggs in the chemical laboratory of the Department of Internal Medicine, Washington University Medical School. The methods were the standard ones used in the laboratory routine for human blood; the methods for inorganics used are those employed in a study of blood plasma (Briggs, 1923) with certain adaptations of standard methods there described.

[3] The chemical determinations of Table II were carried out by E. Ronzoni in the chemical laboratory of the Department of Internal Medicine, Washington University Medical School. The methods were the standard ones for routine human blood determinations.

TABLE I.

*Chemical Constituents of Bee Larval Blood. Mg. per 100 Cc.*

| Sample No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | Averages. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Feeding larva worker. | Feeding larva worker. | Feeding larva worker. | Feeding larva worker. | Feeding larva worker. | Feeding larva worker. | Feeding larva drone. | Feeding larva worker. | Spinning larva worker. | Spinning larva drone. | Pupa drone. | Feeding larva. | Spinning larva and pupa. |
| Non-protein N | | 306 | | 333 | 385 | 335 | 358 | | 312 | 363 | 360 | 340 | |
| Amino N | | 250 | | 286 | 308 | 308 | 294 | | 275 | 296 | 308 | 290 | |
| Urea + $NH_3$ | | Tr. | | Tr. | | Tr. | Tr. | | Tr. | | | | |
| Uric acid | | 6.8 | | 7.7 | | 5.3 | 3.6 | | 3.2 | | | 5.3 | |
| Creatine | | | | | | (1.5) | (2.4) | | (2.7) | | | | |
| Creatinine and creatine | | | | | | (3.1) | | | | | | | |
| Sugar | | | | | | (760) | (790) | | 150 | 120 | 340 | (775) | 203 |
| Total fat | | 587 | | 472 | | | | 370 | | 464 | 374 | 453 | |
| Cholesterol | | 25 | | 33 | | | | 40 | | | | 35 | |
| Lipoid P | | 80 | | 87 | | | | | | 28.5 | 37.0 | 83.5 | 32.7 |
| P | 31 | | 31.6 | | | | | | | | | | |
| Ca | 14.9 | | 14.0 | | | | | | | | | | |
| Mg | 19 | | 22 | | | | | | | | | | |
| K | 95 | | 96 | | | | | | | | | | |
| Na | 11.5 | | 17 | | | | | | | | | | |
| Cl as NaCl | 193 | | | | | | | | | | | | |
| $SO_3$ | Tr. | | | | | | | | | | | | |

TABLE II.

*Chemical Constituents of Bee Larval Blood. Mg. per 100 Cc.*

| Sample No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | Averages. | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Feeding larva worker. | Feeding larva worker. | Spinning larva worker. | Prepupa worker. | Prepupa drone. | Prepupa worker. | Feeding lava worker. | Spinning larva worker. | Feeding larva worker. | Spinning larva worker. | Spinning larva worker. | Feeding larva worker. | Feeding larva. | Spinning larva. | Prepupa. |
| Protein N | 1110 | | 1065 | | | 1055 | 1000 | | 1032 | 1068 | | | | | |
| Protein gm. per 100 cc. (N × 6.25) | 6.94 | | 6.66 | | | 6.60 | 6.25 | | 6.45 | 6.68 | | | | 6.60 (gm. per 100 cc.) | |
| Non-protein N | | 350 | | 325 | | | | | 345 | 342 | | | | 341 | |
| "Albumin" fraction gm. per 100 cc. | | | | | | | | | 3.42 | 3.51 | | | | 3.46 (gm. per 100 cc.) | |
| "Globulin" fraction gm. per 100 cc. | | | | | | | | | 3.03 | 3.17 | | | | 3.10 (gm. per 100 cc.) | |
| Sugar glucose | 688 | (986) | 166 | 166 | 120 | 206 | 684 | 120 | 630 | | 258 | 740 | 685 | 181 | 164 |

472 per cent, sodium 4.4 per cent, chloride 33 per cent, compared with data of (Briggs, 1923) for human plasma. Carbonates are only slightly lower, varying with conditions (Bishop, 1923, *b*).

Excretion of nitrogenous waste is in the form of uric acid, further evidence for which is obtained by examination of the tissues of insect fat bodies (Hollande, 1914), where urates can be detected. Creatinine and creatine values are doubtful, because levulose is present, interfering with the colorimetric reactions of these substances. The values given are certainly too high. Neither amino acid nor fat falls off appreciably during spinning, indicating that energy requirements are satisfied by the glucose present as long as it lasts. The albumin-globulin ratios are not far from mammalian blood figures, and the total protein is just less than that of the mammal.

*B. Osmotic Values of the Constituents of the Blood.*

An attempt was made to prepare a salt solution that would have certain of the properties of this blood, by adding salts in the amounts determined by the above analyses. Since calcium and magnesium chlorides are difficult to dry to definite composition, these metals were added as carbonates, and dissolved by the addition of the correct amount of chloride as HCl. After dissolving the carbonates, the phosphorus was added as acid potassium phosphate, and the remaining potassium and the sodium as bicarbonate. If the less soluble sodium salt was added after the potassium, it refused to go into solution until $CO_2$ was bubbled through for about an hour; but if added before the potassium, solution took place readily. The solution then proved to be saturated with $CO_2$ and if stoppered, remained indefinitely without precipitation. The pH however was far on the acid side of 6.8, the normal pH of bee blood (Bishop, 1923, *b*). When $CO_2$ was blown off in a current of air to a pH of 6.8 a precipitate promptly formed, and the total $CO_2$ of the solution, determined by the Van Slyke technique, was still far too high. It was inferred that organic acids might be present in the blood, and that amino acids and proteins would buffer the fluid; each condition would allow a lower $CO_2$ content at the same pH and $CO_2$ tension. Lactic acid was therefore added as a substitute for these, until the $CO_2$ tension and content were near normal for the blood. The

final mixture was made up as follows (Table III), with or without the lactic acid as desired:

When $CO_2$ was aerated from the mixture containing lactic acid until the pH came to 6.8, the solution contained 25 volumes per cent $CO_2$, which is just below the normal for the blood. At pH 7 the $CO_2$ content was 17 volumes per cent. The former fraction stood overnight without precipitation, the latter precipitated just before the $CO_2$ was reduced to 17 volumes per cent, at pH of about 6.9. On the chart for bee blood prepared on the basis of $CO_2$ content, $CO_2$ tension, and pH (Bishop, 1923, *b*) the point determined by 25 volumes per cent and pH 6.8 is exactly on the curve of the blood, and corresponds to a tension of 43 mm.

TABLE III.

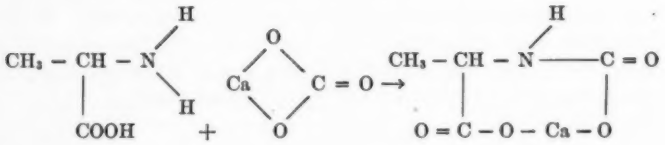
*Concentration of Salts in Synthesized Solution Corresponding to Bee Larval Blood.*

| Material added. | Amounts. | Elemental weights. |
|---|---|---|
| | *mg. per liter.* | *mg. per 100 cc.* |
| $CaCO_3$ | 375 | Ca 15 |
| $MgCO_3$ | 693 | Mg 20 |
| $KH_2PO_4$ | 1360 | P 31 |
| | | K 39 |
| $NaHCO_3$ | 510 | Na 14 |
| $KHCO_3$ | 1430 | K 56 |
| | *cc.* | |
| HCl, N/10 | 328 | Cl 116 |
| Lactic Acid, N/1 | 10 | |

The point 17 volumes per cent at pH 7.0 also falls on the curve, corresponding to a tension of 18 mm. The tensions as measured over the solutions were a little higher than this. The discrepancy is perhaps due to the absence of proteins and amino acids.

The 25 volumes per cent sample tightly stoppered, showed crystals at the bottom of the flask after 24 hours, indicating supersaturation (of calcium diphosphate) at pH 6.8 (Wendt, 1923). It is logical to conclude that considerable calcium and magnesium are present in the solution as organic complexes from which the metals are little ionized. The inorganic figures may therefore give a quite erroneous picture of the metal ion balance. This conclusion is rendered more probable by a consideration of the

large proportion of amino acids in the blood, and that these may react with calcium and magnesium carbonates to form little ionized carbamino compounds (Siegfried, 1905)



With 290 mg. amino N per 100 cc., and 15 mg. Ca and 20 mg. Mg, there are present 17 amino acid groups to each calcium and magnesium atom, a condition which should favor the above reaction. Since the equilibria of these reactions are unknown, nothing can be said of the effective salt ion balance at present except that it is not what it appears to be.

The freezing point of the above solution of pH 6.8 was taken with a Heidenhain thermometer and found $\Delta = 0.24°C$. Without the presence of lactic acid and saturated with $CO_2$ when first made up, $\Delta = 0.26°C$., and with about half or more of the $CO_2$ blown off, $\Delta = 0.22°C$. The freezing point of larval blood was found previously (Bishop, 1923, *a*) to be $\Delta = 0.86°C$., and of pupal blood, $\Delta = 0.75°C$. Assuming the salts of the solution to be ionized, and computing on the basis of the gm. molecular amounts of anions present,[4] this solution, without the lactic acid, and with one-half

[4] This can be done roughly by considering the gm. molecular lowering of the salts in the above mixture. The molecular lowering of the chlorides in dilute solution is 3.5 to 3.7, of acid phosphates, 4.5 (Landolt-Börnstein Tabellen). The lowering for sodium carbonate is 4.7; since the salts are present as bicarbonates, 3.5 was assumed, as only the first dissociation should take place to any extent. This value may be high considering that the salts of calcium were probably less dissociated than assumed. Over half the carbonate is blown off as $CO_2$, so half the value of carbonate added was taken, and computed as bicarbonate. The sum of the quotients of the amount of each salt present, in gm. per liter, divided by the molecular weight, times the respective molecular lowering, gives the computed $\Delta$ of the solution. For the chlorides present, $\Delta = 0.116$, acid phosphates, $\Delta = 0.045$, $\frac{1}{2}$ the bicarbonate, $\Delta = 0.049$, total, $\Delta = 0.240$. Neither the amount of bicarbonate present nor its molecular lowering are precisely known (some $CO_2$ was doubtless lost during the determination of freezing point) so that this value checks fairly well with the measured value of 0.22.

the carbonic acid blown off, figures out $\Delta = 0.24$, isosmotic with 0.37 gm. NaCl per 100 cc. water.

The rest of the osmotic effect should be accounted for by the organic molecules present, the greater portion of which are amino acids. Assuming that the number of molecules of amino acid is somewhat less than the number of amino N atoms, but that the increase in their osmotic effect due to some little ionization compensates for this, we can compute on the basis of a gm. molecular lowering of 1.87 that the amino acid $\Delta = \frac{2.900 \text{ (gm. N per liter)}}{14 \text{ (atomic weight N)}} \times 1.87 = 0.39°C.$ When the lowering of the remaining non-

TABLE IV.

*Comparison of the Known Osmotic Constituents of Human Blood and Bee Larval Blood. Figures in Freezing Point Lowering.*

| Constituents. | Human. | Bee larva. |
|---|---|---|
| Salts | 0.35 | 0.22 |
| $CO_2$ | 0.0475 | (Added above.)* |
| Amino N | 0.03 | 0.39 |
| Other non-protein N | (Above.)† | 0.06 |
| Protein | 0.0033 | 0.003 |
| Glucose | 0.0104 | 0.067 |
| Lactic acid | 0.008 | 0.02 |
| Total | 0.4492 | 0.760 |
| Observed | 0.56 | 0.86 |
| Per cent accounted for | 80 | 88 |

* Computed as amino N.

† Computed as salt.

protein N, presumably equally active, ($\Delta = 0.06$) is added, the total depression becomes 0.45. The amino acids may be partially ionized in the presence of salts, though at pH 6.8 the ionization should be slight, and the value 0.45 may be low. The value for osmotic pressure of plasma proteins in blood (taking the value 30 mm. Hg) in terms of $\Delta$ gives 0.0033 for mammalian blood, and say approximately 0.003 for bee blood. The osmotic depression of the sugar in the larval blood computed on the basis of a gm. molecular lowering of 1.87 is $\Delta = 0.67$, and for pupal blood, 0.0155. Lactic acid, if ionized as a dissociated salt, assuming a gm. molecular lowering of 3.5, would give a $\Delta$ of approximately 0.02 to 0.048

maximal, which is too large, both because the free acid is slightly ionized at 6.8, and because of the probable high error in the determinations of lactic acid in the presence of hydroxy acids.

The freezing point figures that may thus roughly be accounted for by known constituents of this blood sum up as shown in Table IV, where, for comparison, mammalian blood has been figured on the same basis. The constituents as determined account for at least as much of the osmotic effect in bee blood as in mammalian blood. The lowering of the osmotic pressure with pupation in the bee (Bishop, 1923, *b*) is at least in large part accounted for by reduction in sugar content, the increased lactic acid during spinning being compensated for by $CO_2$ blown off. The striking features of the blood in this connection are the small fraction of its total osmotic pressure to be assigned to salts, and the large fraction to amino acids. If any considerable portion of the calcium and magnesium are combined with amino acids, or any amounts of the inorganic acids are bound by organic buffers, the osmotic pressure assigned to the inorganic salts is too large. At any rate it is not more than 25 per cent of the total, as compared to 60 per cent in mammalian blood.

What factors account for the remainder of the osmotic effect is not clear. A solution of amino acids[5] gave a $\Delta$ over twice that computed on the basis of a molecular lowering of 1.87, assuming one molecule of acid for each amino N. The N of this solution was 97 per cent amino N. Other organic products were present,

[5] The amino acids were furnished by F. M. Smith of the Department of Internal Medicine, Washington University School of Medicine. They were prepared from purified milk casein by tryptic digestion according to Dakin's technique, separated from the digestion mixture by extracting with butyl alcohol. This method gives monoamino, monocarboxylic acids, and figuring from the total weight and the amino nitrogen, the molecular weight (average) was 147. The average molecular weight of the amino acids in gliadin and egg albumin weighted according to the amount of each present, turns out to be about 147. The acids employed here, however, in view of their contamination with organic acids and probably with other organic substances containing no amino nitrogen, must have been considerably less than this. It is possible that in considering the amono acids in blood, account should be taken of the fact that the precipitant used (tungstate) leaves fragments higher than amino acids in the filtrate, and the osmotic pressure computed on the basis of one active particle to one amino nitrogen atom will be correspondingly too high.

however, for the pH of the solution was 3, requiring 7.5 cc. $\frac{N}{10}$ alkali to titrate 100 cc. of a solution containing 314 mg. N to neutrality with phenol red. Even allowing for the effect of the titratable acid, the value left is double that computed. We cannot assume complete ionization to account for this, hence still other undetected organic materials were present. Such material might well be present in blood, and adding only 25 per cent to the osmotic effect of the amino acids in bee blood would account for the whole observed value. The presence of inorganic salts might be expected to cause ionization of the amino acids in blood, but these organic salts should be less ionized if anything than the inorganic salts that reacted to form them, with no net increase in activity. On the other hand, if calcium and magnesium form unionized compounds with the amino acids, the resultant osmotic activity would be less than that of the reactants.

In the above consideration of bee blood, the osmotic effect of fats and lipoids has been ignored. If these are largely present in the form of "chilomicrons" as in mammals, this may be justified. If, however, soaps are present, their solution would furnish an appreciable osmotic component. The manner of formation of these fats (presumably from carbohydrates) is uninvestigated, but their presence in the blood itself suggests an intestinal synthesis rather than an origin in the tissues. If the fat is transferred from the intestinal wall through the blood to the fat body cells, soaps might be considered a more labile form for their transfer than combined glycerol esters.

SUMMARY.

1. The blood of the larval honey bee at different stages of development has been subjected to the chemical analyses customary for routine examination of human blood. Certain of both the organic and inorganic constituents vary widely from the mammalian values.

2. Of the organic constituents, sugars are above normal for the mammal during the feeding period, but during pupation reach a level comparable to the mammalian level. Amino acids are 50 times higher, proteins slightly lower, fats higher than in the mammal.

3. Of the inorganics, the sodium and chlorine are very low, potassium, phosphorus, calcium, and magnesium high, and total salt content much lower than in mammalian plasma.

4. Osmotically the amino acids are the most effective fraction, having almost twice the value of the inorganic salts. Changes in sugar content with changed activity account for a large part of the lowering of the osmotic pressure during pupation. Roughly 90 per cent of the osmotic pressure can be accounted for by the constituents detected by analysis.

5. Combination of calcium with amino acids to unionized compounds probably holds part of the calcium in solution, since a prepared salt solution with the analyzed salt concentrations precipitates at the pH of the blood.

6. A solution of amino acids separated from a casein tryptic digest has an osmotic preasure considerably higher than can be accounted for by the known constituents. It is possible that similar metabolites may be present in blood to account for the unexplained fraction of its osmotic pressure.

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# THE EFFECT OF CHANGES IN THE CIRCULATION ON CARBOHYDRATE UTILIZATION.*

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## INTRODUCTION.

The removal of glucose from the blood following massive oral administration of this sugar involves at least the initial stages of carbohydrate metabolism. The rate of its removal, however, may be subject to factors unrelated to carbohydrate metabolism as such.

It is the purpose of this article to record experiments that deal with certain of the factors, both chemical and otherwise, that determine the rate of removal and hence the shape that the blood sugar curve assumes after glucose ingestion. The meaning of the phenomenon of "low sugar tolerance" and its bearing on the pathology of those disorders in which a lowered sugar tolerance is observed depends on an understanding of the factors involved in removing sugar from the blood.

In the normal individual the peak of alimentary hyperglycemia, following administration of 100 gm. of glucose, is usually reached in ½ hour and the blood sugar rarely attains a level in the venous blood higher than 150 mg. per 100 cc. The fall in blood sugar from this peak is rapid, and occurs at a time when glucose is still being actively absorbed from the intestinal tract (1).

* The work here reported is part of a series of studies on chronic arthritis in collaboration with Robert Osgood, M.D., of Boston.

The expenses of this investigation were defrayed by contributions from several sources, including a number of patients.

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Obligation should be expressed to Dr. E. G. Peirce for cooperative assistance in several phases of the work.

In the light of the experimental work of MacLean and de Wesselow (2) and Foster (3), it is difficult to regard the drop in the normal blood sugar curve as other than the result of a stimulus to a sugar-disposing mechanism. The blood sugar, after its initial rise, may fall to hypoglycemic levels and Foster has shown that a second dose of glucose given at this time has little or no effect on the concentration of sugar in the venous blood. These facts are interpreted as indicating that a mechanism capable of rapidly removing sugar from the blood has been stimulated into activity. The observations of Bodansky (4) lend support to this idea, though Folin and Berglund (5) have offered a different explanation for the fall of blood sugar.

It seems clear from recent work that the normal fall of blood sugar cannot be ascribed to oxidation of the sugar. Heat production is definitely increased following the taking of sugar but there is a latent period of ½ to 1 hour in the rise of the respiratory quotient and oxidation of the sugar proceeds for several hours (6). In recent experiments of Linder, Hiller, and Van Slyke (7) only 16 to 27 per cent of the ingested glucose was oxidized in the course of 4 hours.

The blood changes that have been observed during the course of a sugar tolerance test and those that follow the injection of insulin show a remarkable similarity. It seems reasonable to assume that the break in the normal alimentary blood sugar curve is a result of stimulation of insulin production (Foster). After alimentary hyperglycemia, as after insulin, there is a rapid removal of sugar from the blood, evidenced by the fall in blood sugar concentration, often to hypoglycemic levels, which persist for some time (3). A drop in concentration of inorganic phosphates in the blood occurs during the passage of sugar from the blood following glucose ingestion (8) comparable to that which occurs when sugar is driven from the blood by insulin (9).

The observations of Foster (3) on the arterial and venous blood sugar show that during a sugar tolerance test muscle tissue actively takes up sugar from the blood. By analogous experiments, Cori and Cori (10) have recently demonstrated the passage of sugar into muscles following insulin injection. The importance of tissues other than the liver as sites of blood sugar removal is further emphasized by Mann's work. He and Magath (11)

showed by excision of the liver that this organ is not primarily concerned in the hypoglycemic effect of insulin.

The exaggerated and prolonged hyperglycemia observed in individuals with a low sugar tolerance would seem to be referable then, in part, to one or more of the factors concerned in insulin action. Either a delayed stimulation of the pancreas to insulin production or an inadequate output of insulin itself would be productive of a low sugar tolerance. Presumably the latter is the cause of the low tolerance of diabetes.

Another possibility is to be considered however. Since muscle tissue seems to be a prominent site for removal of sugar from the blood, interference with the blood supply to muscle masses might be expected to alter the rate at which sugar leaves the blood.

It is thus conceivable that a lowered sugar tolerance may result from one or more defects in the mechanism that usually operates to remove sugar from the blood. It hardly seems necessary to consider, as some writers do, all cases of low sugar tolerance as indicative of pancreatic defect (12). The diverse disorders in which a lowered sugar tolerance has been reported make it extremely difficult to refer all manifestations of this phenomenon to a single cause.

The value of the alimentary sugar tolerance test has been severely questioned on the ground that the changes in concentration in sugar in the blood represent but a small fraction of that introduced into the stomach. The shape of the blood sugar curve may at times be affected by the rate of intestinal absorption, but any change is usually in the direction of delayed absorption which could not add to the height of the curve. On the other hand experiments will be presented elsewhere (13) indicating that the low sugar tolerance of the type here studied is not referable to abnormally rapid absorption from the intestinal tract.

Pemberton and Foster (14) observed a low sugar tolerance in arthritis and the experiments reported in this paper were conducted as a study of the factors causing the low sugar tolerance in this disorder.

## EXPERIMENTAL.

In all the experiments recorded here the sugar tolerance test was carried out as follows:

After a 12 to 15 hour fast, the subject was given orally 100 gm. of glucose dissolved in 150 cc. of water. Samples of venous blood were obtained from the arm just before the sugar was taken and ½ and 1 hour afterwards. The 1st hour after sugar ingestion seems to be the critical period during which the character of the blood sugar curve is determined. Out of consideration for the subjects the blood sugar was not usually followed beyond this point. Blood sugar was determined by the Folin-Wu method (15).

As will be seen from Table I, the characteristic insulin effect, as evidenced by a fall in blood inorganic phosphorus, follows ingestion of sugar by the arthritic. This lends support to the clinical evidence that this type of low sugar tolerance is other than a failure of insulin production. Inorganic phosphorus was determined by the method of Briggs (16).

The demonstration by Foster (3) that a second dose of glucose, given at a time when the blood sugar is falling after the initial dose, does not cause a second blood sugar rise commensurate with the first, is strong evidence of stimulation of a sugar-disposing mechanism. Experiments analagous to Foster's have been carried out on arthritics and the results are given in Table II. In these experiments the sugar tolerance test was given 1 hour after the ingestion of 50 gm. of sucrose, fed as a lightly flavored peppermint candy, with 100 cc. of water. The rise in blood sugar in venous blood was only a small fraction of that observed in the same individuals when given the test after a 12 hour fast. These results give no evidence of a delayed or inadequate stimulation to the sugar-disposing mechanism in arthritis following this procedure.

The relation between blood supply to muscle tissue on the one hand and the rate of sugar removal from the blood on the other has not been the subject of experimental study, though, as discussed above, the amount of sugar in venous blood is in part determined by the amount removed during passage of blood through muscle tissue.

Pemberton, Hendrix, and Crouter (17) observed, frequently, a marked rise in the oxygen content of venous blood from the arm of the arthritic during the course of a low sugar tolerance test. These results suggest that following sugar ingestion, conditions may be present in the arm tissue of the arthritic, which permit the blood to pass through it with less change in chemical composition than usually takes place. These conditions may be brought about

TABLE I.

*Changes in Content of Blood Sugar and Blood Inorganic Phosphorous Following Glucose Ingestion.*

| Subject. | Date. | Time. | Blood Sugar. | Whole blood inorganic phosphorus. |
|---|---|---|---|---|
| | *1924* | *min.* | *mg. per 100 cc.* | *mg. per 100 cc.* |
| Con. | Feb. 29 | 0 | 89 | 3.45 |
| | | 35 | 138 | 3.52 |
| | | 65 | 194 | 2.85 |
| Sch. | " 21 | 0 | | |
| | | 60 | 210 | 3.73 |
| | | 150 | 202 | 2.88 |
| Kil. | Mar. 19 | 0 | 97 | 3.32 |
| | | 35 | 192 | 3.51 |
| | | 70 | 163 | 3.01 |
| Port.* | " 5 | 0 | 92 | 3.73 |
| | | 35 | 120 | 3.08 |
| | | 65 | 131 | 3.06 |
| Vor. | " 7 | 0 | 96 | 3.22 |
| | | 30 | 164 | 2.61 |
| | | 50 | 158 | 2.74 |
| Seft. | Feb. 26 | 0 | 102 | 3.24 |
| | | 30 | 153 | 2.82 |
| | | 60 | 122 | 2.58 |
| Keen. | June 3 | 0 | 103 | 3.09 |
| | | 60 | 154 | 2.43 |
| | | 120 | 179 | 2.35 |
| | *1925* | | | |
| Kov. | June 23 | 0 | 92 | 3.46 |
| | | 30 | 158 | 2.78 |
| | | 65 | 104 | 2.53 |
| Gra. | " 24 | 0 | 114† | 2.56 |
| | | 30 | 123 | 2.09 |
| | | 70 | 145 | 2.14 |

* Rheumatic fever.

† 50 gm. candy eaten 1 hour before taking glucose.

by areas of closed capillaries or factors accelerating the rate of blood flow. If such vascular conditions are general in muscle tissue, a marked influence on sugar removal from the blood may reasonably be expected. The question as to whether the low sugar tolerance observed in such patients is in any way referable to a

TABLE II.

*Changes in Content of Blood Sugar Following Glucose Ingestion as Influenced by a Prior Dose of Sugar.*

| Subject. | Fasting. | | | 1 hour after ingestion of 50 gm. candy. | | |
|---|---|---|---|---|---|---|
| | Date. | Time. | Blood sugar. | Date. | Time. | Blood sugar. |
| | *1925* | *min.* | *mg. per 100 cc.* | *1925* | *min.* | *mg. per 100 cc.* |
| Giv. | Mar. 20 | 0 | 96 | Mar. 31 | 0 | 74 |
| | | 30 | 160 | | 35 | 94 |
| | | 60 | 179 | | 70 | 115 |
| Tien. | " 30 | 0 | 87 | June 12 | 0 | 144 |
| | | 30 | 170 | | 35 | 163 |
| | | 60 | 176 | | 60 | 165 |
| Hyr. | Apr. 27 | 0 | 99 | " 15 | 0 | 122 |
| | | 30 | 160 | | 30 | 135 |
| | | 60 | 149 | | 60 | 126 |
| Daug. | Jan. 19 | 0 | 95 | " 18 | 0 | 120 |
| | | 30 | 176 | | 30 | 167 |
| | | 85 | 186 | | 60 | 163 |
| Toy. | May 14 | 0 | 99 | " 22 | 0 | 83 |
| | | 30 | 144 | | 30 | 119 |
| | | 65 | 160 | | 60 | 111 |
| Gra. | June 8 | 0 | 95 | " 24 | 0 | 114 |
| | | 30 | 159 | | 30 | 123 |
| | | 60 | 188 | | 70 | 145 |

purely mechanical cause, such as blood supply to muscles would be answered in part could it be demonstrated that the blood sugar curve, after glucose feeding, can be influenced by changes in the blood supply to muscle masses.

Consequently, the sugar tolerance of individuals has been determined at a time when conditions of blood flow through large mus-

TABLE III.

*Effect of Posture on the Blood Sugar Following Glucose Ingestion.*

| Case No. | Subject. | Seated. | | | Legs elevated. | | |
|---|---|---|---|---|---|---|---|
| | | Date. | Time. | Blood sugar. | Date. | Time. | Blood sugar. |
| | | *1925* | *min.* | *mg. per 100 cc.* | *1925* | *min.* | *mg. per 100 cc.* |
| 1 | Pei. | Feb. 10 | 0 | 94 | Feb. 6 | 0 | 94 |
| | | | 35 | 154 | | 30 | 155 |
| | | | 65 | 142 | | 60 | 193 |
| | | | | | " 18 | 0 | 88 |
| | | | | | | 30 | 137 |
| | | | | | | 60 | 169 |
| | | *1924* | | | | | |
| 2 | Ca.* | May 10 | 0 | 99 | " 12 | 0 | 88 |
| | | | 45 | 112 | | 30 | 119 |
| | | | 90 | 109 | | 60 | 104 |
| | | *1925* | | | | | |
| 3 | Wh.* | | | | " 13 | 0 | 86 |
| | | | | | | 35 | 111 |
| | | | | | | 75 | 84 |
| 4 | Sul. | Feb. 19 | 0 | 87 | " 16 | 0 | 90 |
| | | | 30 | 135 | | 30 | 131 |
| | | | 60 | 132 | | 60 | 123 |
| 5 | Mur. | " 20 | 0 | 81 | " 17 | 0 | 90 |
| | | | 30 | 157 | | 30 | 169 |
| | | | 65 | 124 | | 60 | 197 |
| 6 | Ing. | " 28 | 0 | 87 | " 25 | 0 | 94 |
| | | | 30 | 162 | | 35 | 164 |
| | | | 60 | 177 | | 65 | 162 |
| 7 | Hei. | Mar. 19 | 0 | 88 | Mar. 3 | 0 | 87 |
| | | | 30 | 137 | | 30 | 122 |
| | | | 60 | 164 | | 60 | 148 |
| 8 | Shr. | Apr. 25 | 0 | 86 | " 4 | 0 | 91 |
| | | | 35 | 148 | | 30 | 144 |
| | | | 65 | 182 | | 65 | 205 |
| 9 | Fra. | Mar. 10 | 0 | 95 | " 4 | 0 | 88 |
| | | | 30 | 108 | | 35 | 133 |
| | | | 60 | 97 | | 65 | 162 |

* Normal individual.

TABLE III—*Concluded.*

| Case No. | Subject. | Seated. | | | Legs elevated. | | |
|---|---|---|---|---|---|---|---|
| | | Date. | Time. | Blood sugar. | Date. | Time. | Blood sugar. |
| | | *1925* | *min.* | *mg. per 100 cc.* | *1925* | *min.* | *mg. per 100 cc.* |
| 10 | Bro. | Mar. 12 | 0 | 101 | Mar. 5 | 0 | 105 |
| | | | 30 | 149 | | 30 | 161 |
| | | | 60 | 153 | | 60 | 176 |
| 11 | Wy. | " 13 | 0 | 104 | " 6 | 0 | 94 |
| | | | 55 | 168 | | 50 | 156 |
| | | | 95 | 159 | | 85 | 161 |
| 12 | Tay. | " 14 | 0 | 91 | " 9 | 0 | 87 |
| | | | 30 | 156 | | 30 | 155 |
| | | | 60 | 130 | | 60 | 198 |
| 13 | Gif. | " 16 | 0 | 92 | " 11 | 0 | 86 |
| | | | 30 | 150 | | 30 | 152 |
| | | | 65 | 101 | | 60 | 155 |

cle masses had been experimentally altered. The results were compared with the sugar tolerance of the same individuals determined in the usual way.

The subject was placed in a recumbent position on a bed with the legs elevated at right angles to the body, allowing them to rest against the wall for support. One arm was also elevated, being supported by a finger crooked through a loop of bandage attached to a clothes-tree beside the bed. The subject then drank the sugar solution through a straw, and the position was maintained during the course of the experiment. The arm which was not elevated was used for venous puncture. The first blood sample was taken before the legs were elevated. The second and third were taken after assumption of the experimental posture, respectively ½ hour and 1 hour after the ingestion of the sugar. In one case the experiment was continued 1½ hours. Control experiments were carried out on subjects in the experimental posture described above except that no sugar was given.

The results of these experiments and the results of sugar tolerance tests given on the same individuals when seated upright

in the usual way are given in Tables III and IV. Two of the blood sugar curves are presented graphically in Charts 1 and 2.

It will be seen that in a certain proportion of cases (54 per cent) the elevation of the legs has definitely changed a normal or nearly normal blood sugar curve to one characteristic of the low sugar tolerance type.

Attention should be called to the experiments on Cases 13 and 5 which are unusually complete in that data are available showing: the blood sugar curve determined after glucose feeding in the usual position; the effect of the experimental posture on the blood sugar curve following sugar ingestion; and the absence of effect of that posture on fasting blood sugar. Case 1 in the seated position had always a normal tolerance when tested at intervals over a

TABLE IV.

*Control Experiments. Effect of Elevation of the Legs on Fasting Blood Sugar.*

| Case No. | Subject. | Date. | Time. | Blood sugar. | Oxygen capacity. |
|---|---|---|---|---|---|
| | | *1925* | *min.* | *mg. per 100 cc.* | *vol. per cent.* |
| 13 | Gif. | June 9 | 0 | 98 | 16.7 |
| | | | 50 | 94 | 16.6 |
| 5 | Mur. | " 12 | 0 | 98 | 15.8 |
| | | | 47 | 99 | 15.8 |
| 14 | Har. | " 1 | 0 | 90 | 13.9 |
| | | | 50 | 91 | 13.7 |

period of years. When tested a few days after the first posture experiment, she had a normal tolerance. A lowered tolerance was again induced in her some days later by elevation of the legs.

It is to be noted that the blood sugar curve was uninfluenced by posture in two normal persons, or in some arthritics with an already existing low sugar tolerance.

Elevation of the legs for 45 to 50 minutes had no effect on the fasting blood sugar as the results of the control experiment show. This gives no evidence that tissue anoxemia was contributory to the high blood sugar curves obtained under conditions in which anoxemia might occur in the leg tissues. No significant changes in oxygen capacity of the blood were found in the experiments. The blood volume, then, was uninfluenced by the unusual position, the

ingested sugar, or the urine secreted. Physiological effects of the experimental posture including the effects on kidney action will be taken up elsewhere as will the bearing of these results on the pathology of arthritis (13).

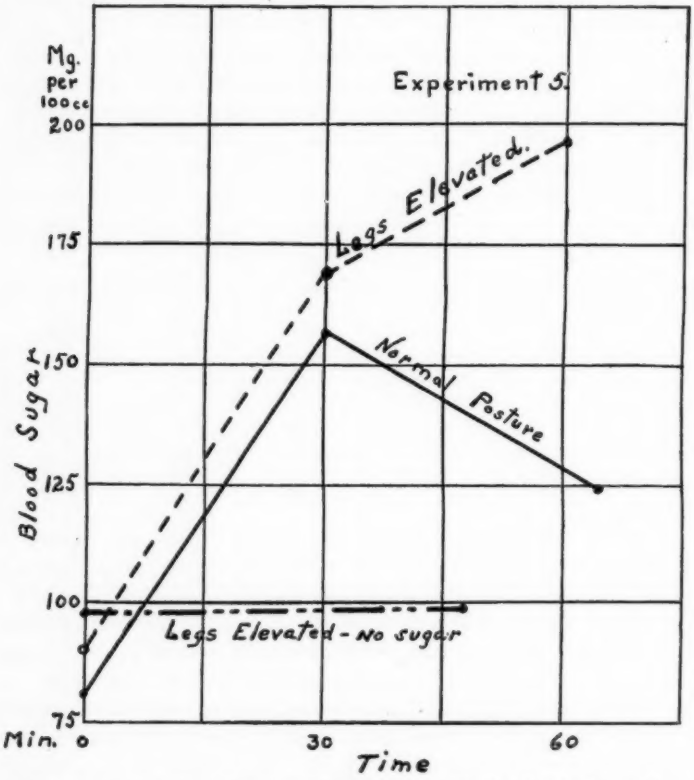


CHART 1. The effect of postural interference with the circulation on the blood sugar curve following glucose ingestion.

It would have been ideal had it been possible to study the sugar curve of arterial and venous blood of one of the elevated limbs, but such studies were obviously not feasible. The positive results are nevertheless of special significance in that they were obtained from blood other than that coming directly from the elevated limbs.

The important point to be emphasized here is the demonstration that the rate at which sugar is removed from the blood can be changed by a procedure which, as far as can be judged at present, is purely mechanical. The chemical processes, regarded as being concerned with sugar removal from the blood, do not seem to be involved. The condition of blood supply and blood flow in the

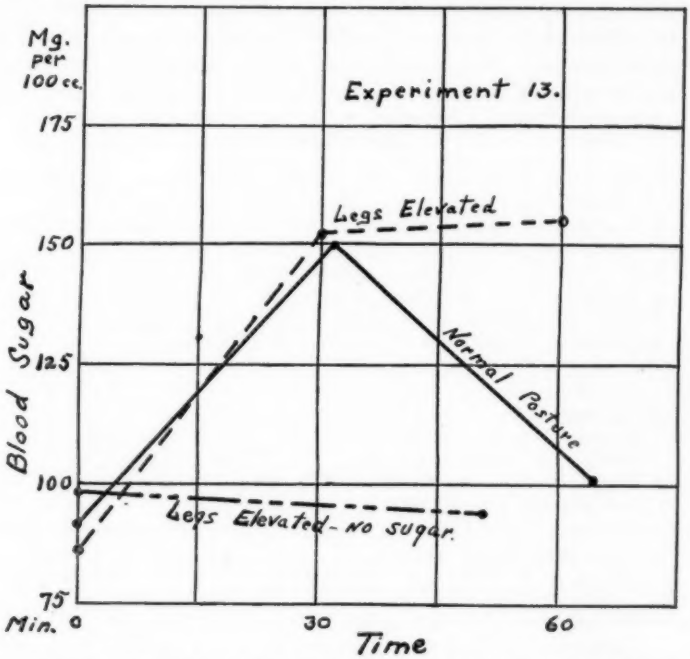


CHART 2. The effect of postural interference with the circulation on the blood sugar curve following glucose ingestion.

tissues should be considered as one factor that may determine the results of sugar tolerance tests.

## SUMMARY.

Some of the factors concerned in determining the rate of sugar removal and hence the shape of the blood sugar curve following alimentary administration of glucose have been discussed.

In a study of the low sugar tolerance of arthritis the influence of certain of these factors has been experimentally studied.

It has been shown that by interfering with the blood supply to large muscle masses through elevation of the legs and one arm, an exaggerated and prolonged hyperglycemia can be induced in some subjects. These facts are interpreted as indicating that the rate at which sugar leaves the blood after glucose ingestion can be influenced by the character of blood supply to tissues active in sugar removal. Purely mechanical factors should be taken into consideration in interpretating the nature of a low sugar tolerance.

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# SOFT PORK STUDIES.

## I. FORMATION OF FAT IN THE PIG ON A RATION MODERATELY LOW IN FAT.

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(Received for publication, August 5, 1925.)

The difference in the firmness of the chilled carcasses of young, immature, pigs and fully developed, mature, hogs when fed a ration of corn and non-softening[1] protein supplement has been one of the outstanding developments of the cooperative soft pork investigations being conducted by the U. S. Department of Agriculture and a number of the State Experiment Stations.[2]

Although variations in the composition and firmness of the fat in hogs of different ages have been reported, the conditions of experimentation were not strictly comparable to those existing in the type of feeding under question. In a study of the Canadian soft bacon problem, Schutt[3] found that most of the bacon classed as soft came from immature or underfed pigs which had received poorly balanced rations such as corn alone or beans alone. The softness was eliminated in the well grown and well finished

* Presented before the American Chemical Society at Baltimore, Maryland, April 6, 1925.

[1] The term non-softening refers to those protein supplements such as tankage, wheat middlings, fish-meal, and skim milk which when fed do not have a tendency to produce a soft fat.

[2] The following were the cooperating stations when the work reported in connection with this paper was under way: Alabama, Arkansas, Georgia, Indiana, Kentucky, Mississippi, North Carolina, Oklahoma, South Carolina, Tennessee, Texas, Coastal Plains Experiment Station, and Iberia Livestock Experiment Farm.

[3] Schutt, F. T., Composition and properties of fat in firm and soft pork, *Canada Exp. Station, Bull. 38*, 1901.

animals after feeding mixed feeds composed of corn, barley, peas, skim milk, and other feeds.

König and Schluckebier[4] observed that as pigs grow older, the melting point of the body fat is raised and that the iodine number is lowered. The weights of the pigs studied as well as the feeds compared do not, however, furnish data strictly comparable with our studies.

Moulton and Trowbridge[5] found in the beef animal that the iodine number of the fat increased and the melting point decreased with age and fatness. The ration fed was composed, in most instances, of corn, linseed meal, and hay. These results seem to indicate that the behavior of the fat in this animal is the reverse of that in the hog when the latter is fed corn and non-softening supplement.

The direct relation between immaturity and softness was first observed in check pigs selected from experimental lots at the start of feeding tests in the soft pork work. These pigs weighed approximately 100 pounds and had received corn with some protein supplement as their main feed. They were usually graded soft or medium soft by the grading committee.[6] The fat of these animals showed rather high iodine numbers and refractive indexes. Control lots fed to a market weight of 200 to 250 pounds on the same ration as the check pigs were graded hard or medium hard with few exceptions and the fat constants were lowered appreciably.

The two experiments reported in this paper were conducted in order to study the question in greater detail. In these experiments, the feed consumption, the composition of the animal body, and the composition of the fat were studied. Periodic killings were made to compare pigs at different stages of development.

[4] König, J., and Schluckebier, J., On the influence of food fat on body fat of swine with special reference to the final location of phytosterin, *Z. Untersuch. Nahrungs- u. Genussmittel*, 1908, xv, 641.

[5] Moulton, C. R., and Trowbridge, P. F., Composition of the fat of beef animals on different planes of nutrition., *J. Ind. and Eng. Chem.*, 1909, i, 761.

[6] Chilled carcasses of all hogs in soft pork experiments are graded by an official committee into five grades; namely, hard, medium hard, medium soft, soft, and oily.

The results of this work along with a summary[7] embracing 371 hogs, on the check and control lots in connection with 4 years of cooperative soft pork experiments, show very strikingly the direct relation of softness to immaturity and also the progressive hardening of the fat of pigs fed on corn and tankage or similar non-softening supplement. The data from the two experiments reported here give an explanation of this behavior. These data show that as the pigs became fatter and better finished, more of the body fat necessarily was derived, through synthesis, from the carbohydrate and protein of the ration and proportionally less from the fat of the ration. The synthetic fat was firm while the ingested fat used for deposition was (soft) oily, and was apparently stored without material change in character.

The present paper records in detail the results of two experiments which deal with the formation of fat in the pig on a ration of corn and skim milk (Experiment A); and corn and tankage with and without alfalfa meal (Experiment B). Special attention is given, in respect to both quantity and quality, to the relationships of the ingested fat and the synthesized fat to the resulting body fat. These relationships are considered to afford a satisfactory explanation of the progressive hardening observed.

## EXPERIMENTAL.

The work, begun in April, 1922, was conducted at the U. S. Experiment Farm, Beltsville, Maryland, as a supplement to the cooperative soft pork investigations. In Experiment A, litters from four sows were used. Of the 34 pigs in the four litters, 27 were actually used in the slaughter tests. Their dams were fed a ration of corn, wheat middlings, and alfalfa hay during the gestation period and after farrowing received a mixed ration of corn, 8 parts; middlings, 4 parts; and fish-meal, 1 part. At the age of 6 weeks, pigs from each litter were killed for analysis. The individual hand feeding of the remaining pigs was started at this point; the ration was corn-meal, 1 part, and skim milk, 3 parts. The pigs continued to suckle their dams until weaned at 10 to 12 weeks of age, when the second killing was made. The

[7] Given in a bulletin, entitled: Some results of soft pork investigations, which is in the course of publication by the cooperating agencies.

ration of corn-meal, 1 part, and skim milk, 3 parts, was fed throughout the test. All of the pigs were fed individually for 2 months after weaning. From that point two litters were lot-fed while the other two continued on individual feeding. Additional killings were made at the approximate weights and ages indicated, as follows: the third at 70 pounds (5 months); the fourth at 100 pounds (6 months); the fifth at 170 pounds (7 months); and the sixth at 225 pounds (8 months).

In Experiment B three litters, numbering 19 pigs, farrowed in October, 1922, were used. The three dams were self-fed corn and wheat middlings on a mixed pasture of sudan grass, soy beans, cow-peas, field-peas, oats, and rape during the gestation period and a mixture of corn-meal, 8 parts; wheat middlings, 4 parts; and fish-meal, 1 part, on rye pasture during the suckling period. At about 4 weeks of age the pigs from two litters were given access to a mixture of corn-meal, 100 parts, and tankage, 15 parts, in a self-feeder in a creep. After weaning at the age of 11 weeks, they received the same mixture in a self-feeder. The total amount of feed consumed by all the pigs was determined by growth stages and the average amount consumed per individual was calculated. In this way any differences in feed utilization due to stage of growth were accounted for and expressed in the feed consumption per unit of gain. The pigs in the third litter were handled in a similar manner, excepting that they were fed individually, on a ration of corn, 100 parts; tankage 14.5 parts; and alfalfa meal, 6 parts. Six killings were made as in the first test.

The composition of the feeds used in the experiments are contained in Table I.

The growth and feeding results as shown by individual pigs are reported in Table II. The pigs are grouped by experiment and by stages of growth. The gains from birth weight to final weight, and the consumption of feed per pound of gain show that the pigs, generally speaking, made satisfactory growth.

*Slaughter.*

In the first and second killings of each experiment, the pigs were taken directly from the feed lot and slaughtered. In the

remaining four killings the pigs were restricted to water for 24 hours prior to slaughter.

All parts of the animal, with the exception of the hair and the contents of the stomach and intestines, were saved for analysis. Weighings were made of live weight, dressed weight, individual and total organs, and blood. A sample of blood was saved for analysis, and the cleaned empty organs were ground and sampled. With the exception of the first two killings, the carcasses were chilled for 48 to 72 hours and graded for firmness.

Back and leaf fat samples were cut out and prepared for fat analysis, after which the carcasses were cut up and separated into three fractions; skin, bone, and meat. The skin and meat were finely ground in a sausage mill and the bones were put through a

TABLE I.

*Composition of Feeds.*

| | Moisture. | Ash. | Protein. | Fat. | Crude Fiber. | Nitrogen-free extract. |
|---|---|---|---|---|---|---|
| Experiment A. | | | | | | |
| Corn | 13.25 | 1.55 | 8.85 | 2.90 | 2.04 | 71.41 |
| Skim milk | 89.97 | 0.62 | 3.78 | 0.30 | — | 5.35 |
| Experiment B. | | | | | | |
| Corn | 14.90 | 1.31 | 8.46 | 3.57 | 1.90 | 69.86 |
| Tankage | 7.40 | 22.90 | 57.12 | 5.70 | 3.24 | 3.64 |
| Alfalfa | 9.11 | 6.95 | 13.60 | 1.75 | 30.00 | 38.59 |

bone cutter. The entire body was thus divided into five fractions; namely, blood, organs, skin, bone, and meat. The sum of these fractions plus the water lost in evaporation from the carcass made up the total analyzed weight. The slaughter weight and the total analyzed weight for each animal are given in Table II. The per cent analyzed weight of the live weight expresses the portion of the animal which was analyzed. This value is less than the live weight by the amount represented by the contents of the digestive tract and the hair.

### *Composition of the Animals.*

The percentage composition of moisture, protein, fat, and ash was determined in each of the fractions referred to above. Com-

TABLE II.

*Growth, Feed Consumption, Slaughter Yield, and Composition of Individual Pigs.*

| Period. | Hog No. and breed.* | Age at slaughter. | Total gain. | Feed consumed per pound gain.† | Slaughter weight. | Total analyzed weight. | Analyzed weight of live weight. | Analyzed weight. | | | | Total weight of fat. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Water. | Protein. | Fat. | Ash. | |
| **Experiment A.** | | | | | | | | | | | | |
| | | *days* | *lbs.* | *lbs.* | *lbs.* | *lbs.* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *lbs.* |
| 1 | 1 H. | 26 | 8.2 | (Weaning | 9.9 | 9.3 | 93.08 | 65.99 | 14.34 | 16.68 | 2.91 | 1.56 |
| | 2 H. | 26 | 9.7 | to close | 11.4 | 10.3 | 90.68 | 65.17 | 15.13 | 16.63 | 3.01 | 1.72 |
| | 3 C. | 30 | 9.2 | of feed- | 12.4 | 11.8 | 94.94 | 65.26 | 13.75 | 18.09 | 2.91 | 2.12 |
| | 4 B. | 41 | 14.4 | ing) | 16.6 | 15.3 | 92.46 | 62.63 | 15.02 | 19.15 | 3.25 | 2.94 |
| | 5 C. | 39 | 17.0 | — | 19.1 | 18.3 | 95.75 | 61.44 | 14.30 | 20.68 | 3.64 | 3.78 |
| 2 | 6 H. | 68 | 19.0 | — | 21.1 | 19.1 | 90.80 | 69.65 | 14.76 | 12.36 | 3.22 | 2.37 |
| | 7 C. | 72 | 25.5 | — | 27.9 | 25.3 | 90.60 | 69.04 | 15.78 | 11.64 | 3.76 | 2.94 |
| | 8 B. | 83 | 28.1 | — | 30.4 | 27.3 | 89.72 | 71.44 | 16.02 | 9.04 | 3.70 | 2.47 |
| | 9 C. | 81 | 29.3 | — | 32.5 | 29.6 | 90.96 | 69.24 | 14.76 | 12.13 | 3.97 | 3.59 |
| 3 | 10 B. | 173 | 50.8 | 4.27 | 52.5 | 47.0 | 89.53 | 69.93 | 16.39 | 10.94 | 3.04 | 5.12 |
| | 11 H. | 158 | 71.0 | 2.77 | 65.5 | 60.8 | 92.82 | 66.48 | 16.43 | 14.28 | 3.00 | 8.72 |
| | 12 C. | 162 | 78.5 | 2.85 | 72.0 | 66.4 | 92.26 | 64.06 | 15.68 | 17.29 | 2.94 | 11.50 |
| | 13 C. | 171 | 78.0 | 2.90 | 77.0 | 71.1 | 92.32 | 66.07 | 16.04 | 14.48 | 3.35 | 10.28 |
| 4 | 14 B. | 201 | 107.0 | 3.76 | 101.0 | 93.9 | 92.94 | 61.40 | 16.51 | 18.74 | 3.15 | 17.59 |
| | 15 C. | 199 | 108.8 | 3.25 | 109.0 | 100.4 | 92.10 | 54.32 | 14.53 | 27.85 | 3.10 | 27.94 |
| | 16 H. | 186 | 107.5 | 3.20 | 109.0 | 101.4 | 93.00 | 58.41 | 15.70 | 22.72 | 2.87 | 23.00 |
| | 17 C. | 190 | 125.0 | 3.57 | 116.0 | 111.0 | 95.70 | 55.28 | 14.68 | 26.95 | 2.74 | 29.91 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5 | 18 H. | 222 | 155.7 | 3.75 | 152.0 | 143.3 | 94.30 | 52.98 | 14.77 | 29.52 | 2.71 | 42.32 |
| | 19 H. | 222 | 166.7 | 3.49 | 160.0 | 151.7 | 94.84 | 50.38 | 14.20 | 32.68 | 2.63 | 49.56 |
| | 20 C. | 226 | 169.5 | 3.99 | 162.0 | 152.3 | 94.02 | 51.37 | 14.16 | 31.81 | 2.67 | 48.44 |
| | 21 C. | 235 | 178.3 | 3.38 | 170.0 | 163.4 | 96.15 | 47.44 | 12.91 | 37.31 | 2.57 | 61.00 |
| 6 | 22 H. | 246 | 204.5 | 3.64 | 193.0 | 185.8 | 96.27 | 46.03 | 13.31 | 38.42 | 2.37 | 71.37 |
| | 23 B. | 261 | 226.0 | 4.20 | 213.0 | 204.6 | 96.08 | 45.44 | 12.96 | 39.21 | 2.35 | 80.25 |
| | 24 C. | 226 | 234.3 | 2.91 | 225.0 | 217.4 | 96.60 | 43.77 | 11.85 | 42.41 | 2.15 | 92.19 |
| | 25 C. | 297 | 228.5 | 4.78 | 228.0 | 217.3 | 95.30 | 40.06 | 11.23 | 46.74 | 2.28 | 101.56 |
| | 26 C. | 250 | 255.5 | 3.55 | 240.0 | 230.3 | 95.96 | 43.20 | 12.21 | 42.37 | 2.20 | 97.56 |
| | 27 H. | 284 | 221.2 | 4.31 | 218.0 | 207.3 | 95.06 | 43.92 | 12.34 | 41.56 | 2.28 | 86.12 |
| | | | | | Experiment B. | | | | | | | |
| 1 | 28 C. | 36 | 14.6 | — | 16.4 | 15.8 | 96.60 | 62.06 | 14.18 | 20.62 | 3.14 | 3.26 |
| | 29 D. | 39 | 15.8 | — | 17.8 | 16.9 | 95.30 | 66.34 | 15.05 | 15.30 | 3.39 | 2.60 |
| 2 | 30 C. | 78 | 33.7 | — | 36.0 | 31.7 | 88.00 | 64.93 | 17.18 | 14.47 | 3.88 | 4.58 |
| | 31 D. | 81 | 43.7 | — | 46.0 | 41.8 | 90.90 | 60.30 | 16.28 | 19.96 | 3.83 | 8.34 |
| | 32 H. | 77 | 36.3 | — | 38.3 | 35.0 | 91.52 | 60.00 | 15.88 | 20.65 | 3.70 | 7.23 |
| 3 | 33 C. | 119 | 74.5 | 2.95 | 73.3 | 69.4 | 94.70 | 60.75 | 14.90 | 21.25 | 3.22 | 14.77 |
| | 34 D. | 122 | 63.2 | 4.50 | 61.8 | 55.0 | 89.07 | 62.67 | 15.98 | 17.63 | 3.85 | 9.70 |
| | 35 H. | 118 | 67.2 | 3.38 | 64.5 | 61.5 | 95.34 | 60.75 | 14.86 | 21.10 | 3.24 | 12.98 |
| 4 | 36 C. | 144 | 115.0 | 2.82 | 110.5 | 104.6 | 94.58 | 53.68 | 13.84 | 29.54 | 2.90 | 30.88 |
| | 37 C | 144 | 111.5 | 2.40 | 104.5 | 100.6 | 96.18 | 52.20 | 13.60 | 31.16 | 3.04 | 31.32 |
| | 38 D | 147 | 103.2 | 2.52 | 98.5 | 90.3 | 91.65 | 58.53 | 15.08 | 22.60 | 3.74 | 20.41 |

TABLE II—*Concluded.*

| Period. | Hog No. and breed.* | Age at slaughter. | Total gain. | Feed consumed per pound gain.† | Slaughter weight. | Total analyzed weight. | Analyzed weight of live weight. | Analyzed weight. | | | | Total weight of fat. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Water. | Protein. | Fat. | Ash. | |
| | | | | Experiment B—*Concluded.* | | | | | | | | |
| | | *days* | *lbs.* | *lbs.* | *lbs.* | *lbs.* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *lbs.* |
| 5 | 39 C. | 216 | 172.5 | 4.19 | 159.5 | 154.9 | 97.14 | 49.39 | 13.80 | 33.70 | 3.04 | 52.26 |
| | 40 C. | 216 | 168.2 | 5.14 | 161.5 | 157.4 | 97.43 | 47.49 | 13.39 | 36.00 | 2.99 | 56.65 |
| | 41 D. | 253 | 180.5 | 3.85 | 172.5 | 160.8 | 93.18 | 47.20 | 12.85 | 36.50 | 3.21 | 57.34 |
| | 42 D. | 253 | 159.5 | 4.50 | 155.5 | 146.9 | 94.45 | 50.21 | 13.75 | 32.82 | 3.14 | 48.20 |
| | 43 H. | 192 | 167.5 | 3.77 | 162.5 | 158.6 | 97.63 | 46.03 | 13.34 | 37.59 | 3.01 | 59.64 |
| 6 | 44 C. | 250 | 230.0 | 3.44 | 225.0 | 218.6 | 97.14 | 43.78 | 12.59 | 40.94 | 2.75 | 89.47 |
| | 45 D. | 219 | 222.0 | 3.03 | 210.0 | 201.6 | 96.02 | 49.21 | 14.26 | 33.25 | 3.25 | 67.04 |
| | 46 H. | 249 | 180.0 | 5.10 | 180.0 | 177.3 | 98.48 | 38.00 | 11.04 | 48.02 | 2.78 | 85.12 |

* The letters attached to the numbers refer to the breed as follows: H., Hampshire; C., Chester White; B., Berkshire; D., Duroc Jersey.

† Skim milk fed in Experiment A is calculated to a grain basis—6 pounds skim milk were considered to be equivalent to one pound of grain.

TABLE III.

*Average Composition of Hogs at Certain Stages of Growth.*

| Stage of growth. | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **Experiment A.** | | | | | | |
| No. of pigs analyzed | 5 | 4 | 4 | 4 | 4 | 6 |
| Live weight at close of experimental period (lbs.) | 13.9 | 28.1 | 70.6 | 114.2 | 170.0 | 231.0 |
| Live weight at slaughter (lbs.) | 13.9 | 28.1 | 66.7 | 108.7 | 161.0 | 219.5 |
| Total weight of parts analyzed (lbs.) | 13.2 | 25.3 | 61.3 | 101.6 | 152.8 | 210.4 |
| Percentage of analyzed weight.* | | | | | | |
| Water | 64.12 | 69.84 | 66.64 | 57.40 | 50.54 | 43.74 |
| Protein | 14.64 | 15.33 | 16.14 | 15.36 | 14.01 | 12.32 |
| Fat | 18.25 | 11.29 | 14.25 | 24.07 | 32.83 | 41.78 |
| Ash | 3.15 | 3.66 | 3.08 | 2.96 | 2.65 | 2.27 |
| Weight (lbs.). | | | | | | |
| Water | 8.46 | 17.68 | 40.86 | 58.35 | 77.25 | 92.08 |
| Protein | 1.93 | 3.88 | 9.90 | 15.62 | 21.41 | 25.90 |
| Fat | 2.41 | 2.86 | 8.74 | 24.47 | 50.20 | 87.95 |
| Ash | 0.42 | 0.93 | 1.89 | 3.01 | 4.05 | 4.77 |
| Weight of fat gained, by periods (lbs.) | | 0.45 | 5.88 | 15.73 | 25.73 | 37.75 |
| Percentage fat gained, by periods | | 3.16 | 13.19 | 36.06 | 46.15 | 61.88 |
| **Experiment B.** | | | | | | |
| No. of pigs analyzed | 2 | 3 | 3 | 3 | 5 | 3 |
| Live weight at close of experimental period (lbs.) | 17.1 | 40.1 | 70.8 | 112.2 | 171.8 | 212.8 |
| Live weight at slaughter (lbs.) | 17.1 | 40.1 | 66.5 | 104.7 | 162.3 | 205.0 |
| Total weight of parts analyzed (lbs.) | 16.3 | 36.2 | 61.8 | 98.4 | 155.7 | 199.2 |
| Percentage of analyzed weight.* | | | | | | |
| Water | 64.20 | 61.74 | 61.39 | 54.80 | 48.06 | 43.66 |
| Protein | 14.62 | 16.45 | 15.25 | 14.17 | 13.43 | 12.63 |
| Fat | 17.96 | 18.36 | 20.00 | 27.77 | 35.33 | 40.74 |
| Ash | 3.26 | 3.80 | 3.44 | 3.23 | 3.08 | 2.93 |
| Weight (lbs.). | | | | | | |
| Water | 10.50 | 22.28 | 40.42 | 57.85 | 79.30 | 90.25 |
| Protein | 2.39 | 5.94 | 10.03 | 14.95 | 22.17 | 26.13 |
| Fat | 2.93 | 6.72 | 12.48 | 27.54 | 54.82 | 80.54 |
| Ash | 0.54 | 1.38 | 2.25 | 3.41 | 5.07 | 6.05 |
| Weight of fat gained, by periods (lbs.) | | 3.79 | 5.76 | 15.06 | 27.28 | 25.72 |
| Percentage fat gained, by periods | | 16.48 | 18.73 | 36.40 | 45.75 | 62.70 |

* Based on the combined results obtained from the separate analyses of blood, organs, meat, bone, and skin.

posite samples of blood were made for hogs of the same size. In several cases the organ and skin samples were handled in the same way. In the analysis of the blood and bones, 300 to 400 gm. samples were given a preliminary drying, weighed, and then ground in a pulverizing mill. Whenever the organ and skin samples were found to be too coarsely ground, they were treated

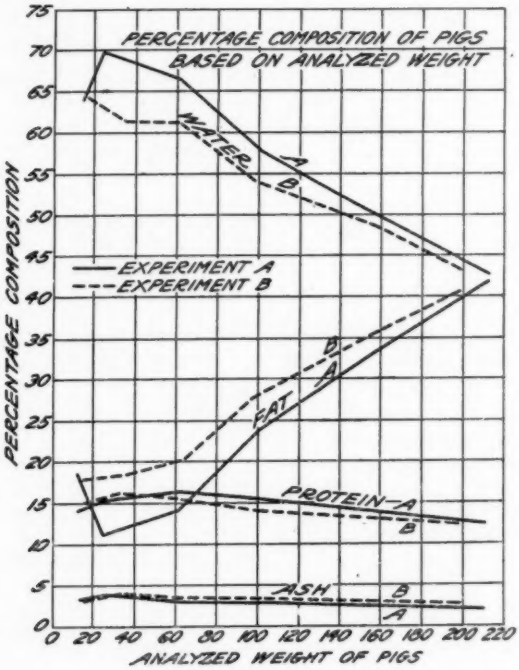


CHART 1.

in a similar manner. The methods of analysis of the Association of Official Agricultural Chemists were followed throughout. Triplicate determinations were made on sufficiently large samples to insure reliable results. From these analyses, the composition of each animal was calculated back to the total analyzed weight. The per cent of water, protein, fat, and ash based upon the analyzed weight and also the total weight of fat are given in Table

II. There was some variation within a group of animals, on the other hand, with the number of animals used, and with one experiment a check on the other, the average figures given in Table III can be taken as representative of the conditions in the experiments.

Chart 1 shows the change in percentage composition of the pigs on the analyzed weight basis. The most noteworthy point in this chart is the rise in fat content. The pigs in Experiment B were fatter throughout the trial than those in Experiment A although the latter reached a heavier weight and consequently a fatter condition at the close of the experiment.

*Composition of the Fat.*

Fat samples were prepared from the meat fractions of each pig in addition to the back and leaf samples previously mentioned. These were usually prepared by rendering the meats on the steam bath, then decanting and filtering the fat. This sample, which was termed the meat fat, represented 80 to 90 per cent of all the fat in the animal. The general analysis on the back, leaf, and meat fat samples from the individual pigs consisted in the determination of the refractive index, iodine number, and melting point as shown in Table IV. Included are the Soft Pork Committee[6] grades. The change in these fat constants, indicating a decrease in the degree of unsaturation of the fat, was accompanied by the change in carcass grades from soft to hard. The small pigs under 100 pounds were soft, while the 165 to 225 pound pigs were hard. It will be noted that in Experiment B, the weanling pigs of the second killing were the softest. Otherwise all show the progressive hardening step by step. The corn and skim milk ration in the first trial resulted in the formation of a slightly harder fat than the corn and tankage ration of Experiment B. The features of these results are shown in Chart 2 in which the changes in the refractive index and iodine number of the meat fat are plotted. These results on fat constants and committee grades confirm the conclusions previously referred to, that as hogs take on weight and finish they become progressively harder.

After completing the analyses on the fat samples of the individual pigs, aliquot samples of meat fat for each stage of growth

TABLE IV.

*Grades and Fat Constants of Individual Pigs.*

| Stages of growth. | Hog No. | Grade. | Refractive index—40°C. | | | Iodine No. | | Melting point. | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Meat. | Back. | Leaf. | Meat. | Back. | Meat. | Back. |
| **Experiment A.** | | | | | | | | | |
| 1 | 1 | — | 1.4602 | — | — | 73.7 | — | 25.5 | — |
| | 2 | — | 1.4598 | — | — | 72.4 | — | 26.1 | — |
| | 3 | — | 1.4606 | — | — | 73.6 | — | 26.9 | — |
| | 4 | — | 1.4609 | — | — | 72.4 | — | 27.7 | — |
| | 5 | — | 1.4604 | — | — | 73.0 | — | 27.5 | — |
| 2 | 6 | — | 1.4604 | — | — | 71.5 | — | 27.3 | — |
| | 7 | — | 1.4603 | — | — | 70.9 | — | 27.3 | — |
| | 8 | — | 1.4606 | — | — | 73.5 | — | 27.7 | — |
| | 9 | — | 1.4604 | — | — | 71.5 | — | 26.7 | — |
| 3 | 10 | Soft. | 1.4604 | 1.4602 | — | 69.6 | 73.9 | 27.8 | 28.0 |
| | 11 | " | 1.4600 | 1.4594 | — | 68.4 | 69.9 | 28.0 | 28.6 |
| | 12 | " | 1.4597 | 1.4592 | — | 65.9 | 66.0 | 29.0 | 31.0 |
| | 13 | " | 1.4596 | 1.4594 | — | 64.0 | 65.6 | 33.2 | 35.8 |
| 4 | 14 | " | 1.4597 | 1.4601 | — | 65.9 | 68.0 | 31.0 | 31.6 |
| | 15 | " | 1.4591 | 1.4592 | — | 61.6 | 61.5 | 31.4 | 31.4 |
| | 16 | " | 1.4594 | 1.4595 | — | 63.0 | 65.2 | 29.4 | 30.8 |
| | 17 | " | 1.4592 | 1.4592 | — | 62.1 | 61.8 | 30.8 | 32.4 |
| 5 | 18 | Med. hard. | 1.4590 | 1.4590 | 1.4580 | 61.0 | 60.6 | 34.0 | 37.0 |
| | 19 | Hard. | 1.4591 | 1.4588 | 1.4579 | 60.8 | 60.5 | 34.2 | 38.2 |
| | 20 | " | 1.4589 | 1.4586 | 1.4579 | 60.1 | 57.1 | 36.0 | 39.8 |
| | 21 | " | 1.4585 | 1.4585 | 1.4577 | 56.3 | 56.3 | 37.6 | 40.2 |
| 6 | 22 | " | 1.4586 | 1.4584 | 1.4577 | 60.0 | 59.4 | 30.8 | 37.4 |
| | 23 | " | 1.4584 | 1.4584 | 1.4576 | 58.9 | 58.6 | 33.0 | 36.4 |
| | 24 | " | 1.4586 | 1.4584 | 1.4580 | 57.4 | 56.3 | 36.2 | 38.6 |
| | 25 | " | 1.4589 | 1.4589 | 1.4580 | 59.0 | 59.1 | 38.5 | 38.5 |
| | 26 | " | 1.4584 | 1.4582 | 1.4575 | 57.7 | 58.0 | 39.2 | 39.2 |
| | 27 | " | 1.4590 | 1.4589 | 1.4583 | 60.1 | 61.2 | 36.1 | 36.1 |
| **Experiment B.** | | | | | | | | | |
| 1 | 28 | — | 1.4605 | — | — | 73.9 | — | 26.1 | — |
| | 29 | — | 1.4600 | — | — | 69.8 | — | 30.1 | — |
| 2 | 30 | — | 1.4609 | — | — | 80.4 | — | 21.7 | — |
| | 31 | — | 1.4604 | — | — | 75.7 | — | 26.9 | — |
| | 32 | — | 1.4605 | — | — | 76.9 | — | 25.1 | — |

TABLE IV—*Concluded.*

| Stages of growth. | Hog No. | Grade. | Refractive index—40°C. | | | Iodine No. | | Melting point. | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Meat. | Back. | Leaf. | Meat. | Back. | Meat. | Back. |
| Experiment B—*Concluded.* | | | | | | | | | |
| 3 | 33 | Soft. | 1.4600 | — | — | 70.3 | — | 35.9 | — |
| | 34 | " | 1.4610 | — | — | 78.2 | — | 27.7 | — |
| | 35 | " | 1.4602 | — | — | 73.0 | — | 30.4 | — |
| 4 | 36 | Med. hard. | 1.4596 | 1.4598 | 1.4590 | 67.7 | — | 30.1 | — |
| | 37 | " soft. | 1.4591 | 1.4593 | 1.4582 | 63.2 | — | 37.5 | — |
| | 38 | Soft. | 1.4599 | 1.4602 | 1.4590 | 69.7 | — | 38.1 | — |
| 5 | 39 | Hard. | 1.4593 | 1.4593 | 1.4586 | 64.6 | — | 36.4 | — |
| | 40 | " | 1.4591 | 1.4591 | 1.4581 | 63.2 | — | 40.0 | — |
| | 41 | Med. Hard. | 1.4589 | 1.4590 | 1.4581 | 61.8 | — | 40.0 | — |
| | 42 | Hard. | 1.4589 | 1.4590 | 1.4583 | 60.7 | — | 39.9 | — |
| | 43 | " | 1.4594 | — | — | 64.8 | — | 37.6 | — |
| 6 | 44 | Med. hard. | 1.4589 | 1.4589 | 1.4580 | 61.3 | — | 39.6 | — |
| | 45 | Hard. | 1.4591 | 1.4592 | 1.4584 | 63.3 | — | 39.4 | — |
| | 46 | " | 1.4589 | 1.4591 | 1.4584 | 62.2 | — | 39.4 | — |

in each feeding test were combined in a composite sample which was used for a detailed study of the fat composition. Most of the fat constants, Table V, were included in this study along with the separation of the fatty acids by the lead salt-ether method. For the fat constants, the methods of the Association of Official Agricultural Chemists[8] were followed, while for the lead salt-ether method the directions given by Lewkowitsch[9] and by Jamieson and Baughman[10] were used. In addition to determining the percentage of saturated and unsaturated acids present, the percentage composition of the individual unsaturated acids was also determined. The unsaturated fraction was brominated and the ether-insoluble bromides separated from the ether-soluble. The former yielded no hexabromide (linolenic acid)

[8] Official and tentative methods of analysis, *Assn. Off. Agric. Chem.*, Washington, 1920.

[9] Lewkowitsch, J., Chemical technology and analysis of oils, fats, and waxes, London, 6th edition, 1921–23, iii.

[10] Jamieson, G. S., and Baughman, W. F., *Cotton Oil Press*, 1922, vi, 41.

but was insoluble in boiling benzene and did not melt at 180°C., thus indicating an octabromide, which probably belonged to the clupanodonic acid series. The ether-soluble material was further tested to demonstrate the presence of both linolic and oleic acids. The actual percentages of these acids were calculated from the iodine number of the mixture of these two acids.

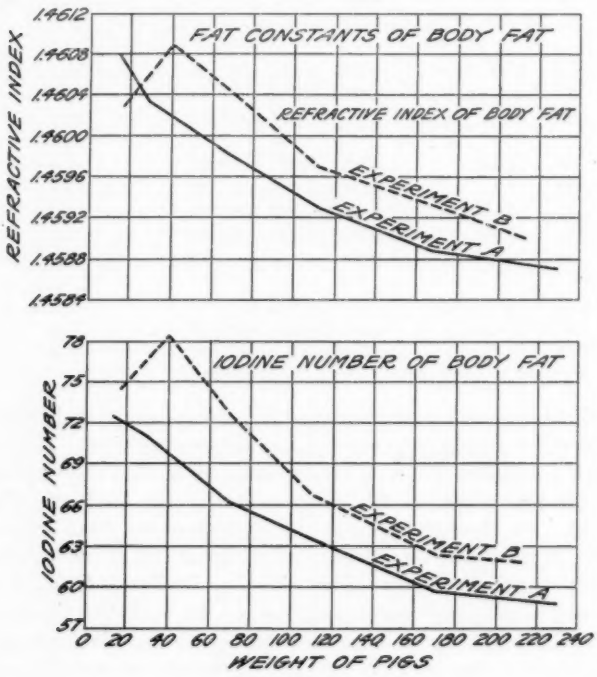


CHART 2.

The results of the fat analysis of the composite samples are shown in Table V. The refractive index, iodine number, and melting point values, already indicated, show increasing hardness with increasing size, with the exceptions noted. The refractive index and iodine number are shown in Chart 2. The specific gravity, saponification number, the proportion of insoluble acids, the Reichert-Meissl number, the Polenske number, and the titer

TABLE V.

*Results Obtained from the Analysis of Composite Meat Fats, Giving the Composition of the Average Fat at Definite Stages of Growth.*

| Stage of growth. | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **Experiment A.** | | | | | | |
| Refractive index | 1.4608 | 1.4603 | 1.4598 | 1.4593 | 1.4589 | 1.4587 |
| Iodine No. | 72.5 | 71.0 | 66.1 | 63.2 | 59.7 | 58.8 |
| Melting point | 25.5 | 26.7 | 35.1 | 37.1 | 38.7 | 37.5 |
| Specific gravity | 0.9043 | 0.9053 | 0.8980 | 0.8970 | 0.8956 | 0.8957 |
| Saponification No. | 200.0 | 199.4 | 194.6 | 196.1 | 196.0 | 195.0 |
| Insoluble acids, per cent. | 94.36 | 92.65 | 94.30 | 95.51 | 95.85 | 95.61 |
| Reichert-Meissl No. | 0.58 | 1.46 | 0.94 | 1.22 | 1.10 | 1.03 |
| Polenske No. | 1.25 | 1.37 | 2.56 | 4.31 | 4.90 | 5.19 |
| Titer test | 37.0 | 37.5 | 37.8 | 37.9 | 39.2 | 39.1 |
| Unsaturated acids, per cent. | 62.0 | 63.5 | 59.3 | 59.8 | 58.7 | 58.8 |
| Saturated acids, per cent. | 30.7 | 29.0 | 34.6 | 35.6 | 36.5 | 36.9 |
| Total acids, per cent. | 92.7 | 92.5 | 93.9 | 95.4 | 95.2 | 95.7 |
| Iodine No.—unsaturated acids | 107.7 | 109.3 | 107.5 | 103.1 | 101.0 | 100.8 |
| Oleic acid, per cent. | 50.4 | 50.5 | 48.2 | 51.3 | 51.7 | 52.0 |
| Linolic acid, per cent. | 11.3 | 12.7 | 10.9 | 8.4 | 6.9 | 6.7 |
| Octabromide acid, per cent. | 0.25 | 0.23 | 0.16 | 0.09 | 0.06 | 0.07 |
| **Experiment B.** | | | | | | |
| Refractive index | 1.4603 | 1.4609 | 1.4604 | 1.4597 | 1.4593 | 1.4590 |
| Iodine No. | 74.6 | 78.5 | 72.8 | 67.0 | 62.7 | 62.2 |
| Melting point | 27.2 | 23.8 | 30.4 | 36.6 | 39.4 | 39.8 |
| Specific gravity | 0.8983 | 0.8986 | 0.8984 | 0.8961 | 0.8960 | 0.8963 |
| Saponification No. | 198.2 | 197.1 | 196.6 | 196.6 | 197.2 | 195.8 |
| Insoluble acids, per cent. | 95.75 | 95.50 | 95.49 | 95.50 | 95.57 | 95.49 |
| Reichert-Meissl No. | 0.90 | 1.42 | 2.29 | 1.26 | 0.67 | 0.82 |
| Polenske No. | 3.25 | 4.65 | 4.97 | 4.52 | 3.96 | 3.53 |
| Titer test | 37.0 | 34.0 | 36.4 | 37.9 | 38.9 | 39.3 |
| Unsaturated acids, per cent. | 63.8 | 67.7 | 63.4 | 61.8 | 58.7 | 58.9 |
| Saturated acids, per cent. | 32.0 | 27.9 | 31.7 | 33.7 | 36.7 | 36.6 |
| Total acids, per cent. | 95.8 | 95.6 | 95.1 | 95.5 | 95.4 | 95.5 |
| Iodine No.—unsaturated acids | 111.4 | 112.7 | 113.7 | 105.6 | 104.8 | 104.3 |
| Oleic acid, per cent. | 49.2 | 51.2 | 47.2 | 51.4 | 49.4 | 49.8 |
| Linolic acid, per cent. | 14.4 | 16.2 | 16.1 | 10.3 | 9.3 | 9.1 |
| Octabromide acid, per cent. | 0.21 | 0.24 | 0.14 | 0.09 | 0.07 | 0.07 |

test show only slight fluctuations with the other fat constants. It could hardly be expected that these values would show much change under the conditions of this experiment. There is a slight

drop in specific gravity and saponification number. The Reichert-Meissl number is constant (within experimental error) throughout. The Polenske number shows some fluctuation; in Experiment A it increases as the animal increased in size while in the second experiment it is at a maximum in the 70 pound size. The titer test shows a slight rise in Experiment A and a more decided

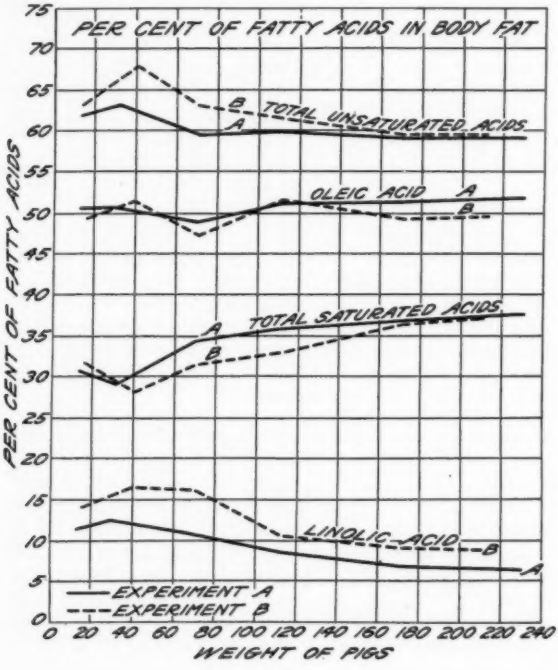
PER CENT OF FATTY ACIDS IN BODY FAT
PER CENT OF FATTY ACIDS
TOTAL UNSATURATED ACIDS
OLEIC ACID
TOTAL SATURATED ACIDS
LINOLIC ACID
EXPERIMENT A
EXPERIMENT B
WEIGHT OF PIGS
0 20 40 60 80 100 120 140 160 180 200 220 240
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75

CHART 3.

change in Experiment B. As is to be expected this determination gives values which are comparable with the changes in the refractive index, iodine number, and melting point.

The total saturated and the unsaturated fatty acids (see Chart 3) are of particular interest. In both trials, the lowest per cent of saturated acids occurred at the weanling stage and the highest per cent at the fifth and sixth stages which were very close; the

total unsaturated acids were the reverse of this. Of the individual unsaturated acids the oleic acid was surprisingly constant at about 50 per cent of the fat. Linolic acid, however, decreased as the saturated acids increased; in the first trial it declined from a high point of 12.7 per cent in the second killing to 6.7 per cent in the last killing, while in the second trial the percentages were 16.2 and 9.1, respectively. These declines, amounting to nearly

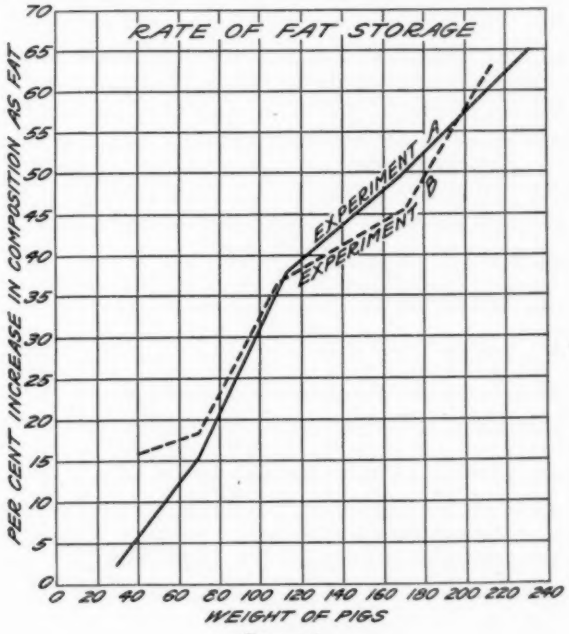


CHART 4.

50 per cent in each case, were considered very significant, particularly since they paralleled the change from soft to hard in the body fat. No attempt was made to separate or to determine quantitatively the different saturated acids of these twelve samples. Certain saturated acid fractions were, however, examined and found to consist of palmitic, stearic, and myristic acids; occurring in decreasing amounts in the order named, the latter being relatively small.

It has long been known that in a fattening animal the gain in fat makes up a much higher proportion of the total gain than it does in a growing animal. From the average composition of

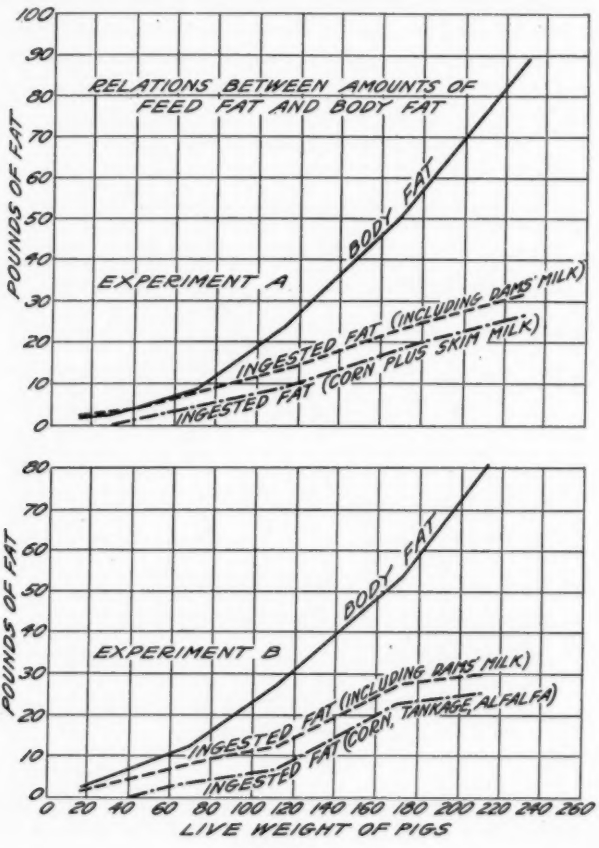
RELATIONS BETWEEN AMOUNTS OF FEED FAT AND BODY FAT
POUNDS OF FAT
EXPERIMENT A
BODY FAT
INGESTED FAT (INCLUDING DAMS' MILK)
INGESTED FAT (CORN PLUS SKIM MILK)
EXPERIMENT B
BODY FAT
INGESTED FAT (INCLUDING DAMS' MILK)
INGESTED FAT (CORN, TANKAGE, ALFALFA)
0 20 40 60 80 100 120 140 160 180 200 220 240 260
LIVE WEIGHT OF PIGS

Chart 5.

the hogs (see Table III), the percentage of fat increase by periods has been calculated for both trials. This value was obtained by dividing the gain in fat between two given periods by the gain in live weight for the same interval. In the first experiment

there was an increase from 3.16 to 61.88 per cent while in the second experiment the increase was from 16.48 to 62.70 per cent. These figures are shown graphically in Chart 4. Knowing that the feed fat, when the supply of total nutrients is in excess of the maintenance requirements, will be utilized for the formation of body fat, it is easy to see that there will be a wide variation in the ratio of feed fat consumed to body fat deposited per pound of gain. A 30 pound pig will consume about 3 pounds of feed, while a 225 pound pig will consume about 5 pounds of feed for each pound of gain. If this feed contained 4 per cent of fat, the amount of ingested fat would be in the one case 0.12 pounds and in the other 0.20 pounds. However, on the basis of the percentages of fat increases given above, the 30 pound pigs deposited only 0.03 to 0.17 pounds of fat for a gain of one pound and the 225 pound pigs deposited 0.61 to 0.63 pounds of fat. Thus, on a moderate amount of fat in the diet, the body fat in the small pig may be derived very largely from the ingested fat. In the larger animal, the amount of feed fat supplied makes up a much smaller percentage of the body fat deposited.

It is apparent that the conditions outlined above existed in the experiments under discussion. The actual amounts of the feed fat consumed have been calculated for the six groups in each of the two experiments. These figures together with the amounts of body fat as given in Table III were plotted (see Chart 5) to show the relationship between the feed fat and the body fat. Although no actual figures were available to show the amount of fat derived from the dam's milk, it was estimated that the average weight of milk fat consumed per pig from birth to weaning was 4 pounds in Experiment A, and 4.5 pounds in Experiment B.[11] These amounts were added to the total ingested fat and the resulting figures plotted to show the total fat consumption.

The curves clearly show the widening of the ratio between ingested fat and body fat. Thus with the growth of the animal, more and more of the body fat necessarily was synthesized. The only time when the feed fat could have met the demands of the body was during the suckling period. Of course, not all of the ingested fat was assimilated and used for fat storage but the

[11] Henry, W. A., and Morrison, F. B., Feeds and feeding, Madison, 18th edition, 1923, 698.

percentage was probably quite constant throughout so that the relative change in ratio was likewise constant.

These changes immediately suggest a close relationship to the change in firmness of the fat. It is common knowledge, fully borne out by results obtained in the soft pork work, that ingested fat is deposited in the animal with but slight modification of the fatty acid make-up. Corn oil is composed largely of the unsaturated acids, oleic and linolic, and since this is the predominating fat (oil) in the rations in question, the acids mentioned would predominate in the ingested fat and would be expected to result in the formation of a soft, oily fat. Since the fat formed in the animal body from carbohydrate and protein (that is synthetic fat) is usually hard, the character of the fat present in the adipose tissues of the pig at a particular stage of development would be dependent, other things being equal, on the ratio of body fat

TABLE VI.

*Composition of Feed Fat Expressed as the Per Cent Fatty Acids of Total Fat.*

| | Saturated. | Oleic. | Linolic. |
|---|---|---|---|
| Corn oil.[12] | 11.2 | 43.4 | 39.1 |
| Butter fat.[9] | 55.0 | 35.0 | — |
| Tankage | 27.0 | 59.5 | 6.0 |

derived from ingested fat to that derived synthetically. Some information on this point is furnished by the following study of the fatty acid content of the feed fat and the body fat.

*A Comparison of the Fatty Acids Ingested with Those Deposited.*

The comparison of the amounts of fatty acids ingested with the total fatty acids deposited was next made. Since no figures on the composition of the fat of sow's milk were available, the comparison was based on the results from weaning to slaughter. In the case of the fatty acids in the body fat, the amount of each present at weaning was subtracted from that present at each following stage.[13]

[12] Baughman, W. F., and Jamieson, G. S., *J. Am. Chem. Soc.*, 1921, xliii, 2696.

[13] The formation of fat during the suckling period will be given further study.

The three fractions of the fat considered were the total saturated acids, oleic acid, and linolic acid. The figures used for calculating the composition of the feed fat are given in Table VI. Al-

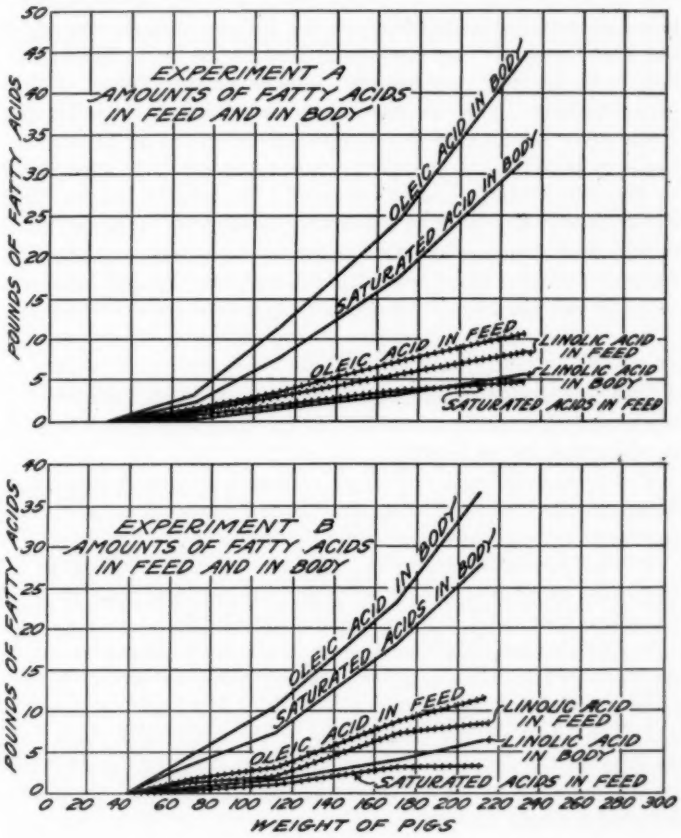
EXPERIMENT A
AMOUNTS OF FATTY ACIDS IN FEED AND IN BODY
POUNDS OF FATTY ACIDS
OLEIC ACID IN BODY
SATURATED ACID IN BODY
OLEIC ACID IN FEED
LINOLIC ACID IN FEED
LINOLIC ACID IN BODY
SATURATED ACIDS IN FEED
EXPERIMENT B
AMOUNTS OF FATTY ACIDS IN FEED AND IN BODY
POUNDS OF FATTY ACIDS
OLEIC ACID IN BODY
SATURATED ACIDS IN BODY
OLEIC ACID IN FEED
LINOLIC ACID IN FEED
LINOLIC ACID IN BODY
SATURATED ACIDS IN FEED
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300
WEIGHT OF PIGS

CHART 6.

falfa was omitted from consideration because of its small fat content.

The total saturated acids and the oleic acid ingested and deposited were comparable to the whole fats (see Chart 5) in

that the amounts in the body were in excess of the supply in the feed. The linolic acid, however, presents a strikingly different situation since the total amount ingested was in excess of that in the body in late as well as early growth. From unpublished data on the composition of hog fats on various rations, it has been observed that the linolic acid in the body follows the supply in the feed very closely. The actual percentage of this acid has been found to vary through extremely wide limits. Apparently linolic acid is not as essential a factor in adipose tissue as is oleic acid, since the amount appears to be controlled by the amount furnished in the feed. The drop in the per cent of linolic acid in the fat with a corresponding rise in the per cent of saturated acids explains the hardening of the fats in the experiments reported. Linolic acid has an extreme softening influence and the saturated acids, of course, have the opposite effect.

SUMMARY.

The progressive hardening of hogs on a ration containing a moderately low amount of softening fat such as is found in corn is accounted for as follows:

1. The change from a soft fat as found in young, immature hogs to a hard fat in older, mature hogs was accompanied by an increased rate of fat deposition.

2. This increased rate of fat deposition caused a widening of the ratio of body fat derived from carbohydrate and protein (hard) to that derived from ingested fat (soft).

3. The change in the composition of the body fat resulted in a decrease in the iodine number and refractive index and an increase in the melting point.

4. The effect on the proportions of the fatty acids were: an increase in the per cent of total saturated acids and a decrease in the per cent of linolic acid with the per cent of oleic acid remaining nearly constant.

# THE PROPERTIES AND COMPOSITION OF OOCYTIN. II.*

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(Received for publication, August 26, 1925.)

In resuming the experimental work with oocytin it was hoped that the procedure used by Clark[1] might be so modified that a more uniform product would be obtained and that it would be possible to more definitely determine the chemical and physiological properties of this peculiar substance.

After a number of preliminary experiments it was possible to prepare oocytin by at least three methods which are described below in the order of their importance.

The first or "acid method" is essentially that used by Clark.[1] The defibrinated blood is kept in a refrigerator (11°C.) until thoroughly cool and is then centrifuged. (Defibrinated blood may be kept in this manner 12 to 16 hours without decreasing the yield of oocytin.) 100 cc. of 10 per cent barium chloride solution are added with constant stirring to each liter of clear serum. This mixture is also kept in a cool place for 24 hours, after which time it is centrifuged at a low speed for about 5 minutes and the precipitate is washed several times with 2 per cent barium chloride solution to remove all traces of serum. One tenth normal hydrochloric acid is then added to the washed precipitate, using 50 cc. of acid for each liter of serum. After a thorough stirring the mixture is put into the refrigerator (11°C.) for 12 to 24 hours, after which time it is well centrifuged. To the slightly opalescent liquid, which is decanted from the precipitate, is slowly added an equal volume of 0.1 N sulfuric acid. This acid mixture, after being well stirred, is placed in the incubator (35–40°C.) for 24 hours and again thoroughly centrifuged to remove the barium sulfate. To the opalescent fluid thus obtained four to five volumes of acetone (boiling point 56–57°C.)

* Aided by a grant from the Research Board of the University of California.

† The work reported in this paper is an abstract of a thesis submitted by Paul W. Sharp in partial fulfillment of the requirements for the degree of Master of Science in the Graduate School of the University of California.

[1] Clark, G. W., *J. Biol. Chem.*, 1918, xxxv, 253.

are added and the mixture is placed in the refrigerator for 20 to 24 hours. The acetone-acid mixture is then siphoned off, the precipitate collected on a hardened filter paper and washed several times with each of the following: (1) 95 per cent alcohol, (2) absolute alcohol, and (3) anhydrous ether. The ether-moist precipitate is put into an incubator containing sulfuric acid and dried for 48 hours at 38°C. The finely powdered white substance is kept in a tightly stoppered container and protected from light.

Since oocytin is insoluble in dilute alkali it was thought that it might be possible to precipitate the substance from a neutral solution. This formed the basis for the second or "neutral method" of preparing oocytin. Previous to precipitation with acetone the procedure is the same as that outlined under the "acid method" just described.

The opalescent fluid is carefully neutralized with 0.1 N sodium hydroxide—adding sufficient alkali to adjust the reaction to a pH of approximately 8.0 (phenol red). The precipitate which results from neutralization settles rapidly (the flocculi being more dense by this method) and is washed and dried in the usual manner.

Repeated experiments showed that this method did not give the maximum yield of oocytin, and in certain cases only a very small portion was obtained. If acetone is added to the clear, alkaline solution more oocytin is precipitated, but not until four to five volumes of acetone have been added.

The third or "carbon dioxide method" is carried out as follows: The precipitate, after being thoroughly washed with 2 per cent barium chloride, is suspended in distilled water (100 cc. of water for each liter of serum) and a slow stream of carbon dioxide is passed into the mixture for an hour or more. After standing several hours the mixture is centrifuged and four to five volumes of acetone are added to the slightly opalescent liquid. The yield of oocytin by this method is also much less than that obtained by the acid method.

Since there are no available chemical tests for oocytin it was necessary to determine the relative purity of the various samples by testing their action in producing fertilization membranes on the eggs of matured sea urchins (*Strongylocentrotus purpuratus*). After several trials it was found that when the sea urchins were shipped to Berkeley (*via* express or automobile) they were not desirable for this type of experimentation. Eggs tested at the

Hopkins Marine Laboratory[2] (Pacific Grove, California) immediately after collecting formed 100 per cent membranes with sperm in less than 2 minutes while if shipped to Berkeley only 25 to 35 per cent membranes were formed in 5 minutes and rarely over 50 per cent at any time.

As soon as the sea urchins were collected they were taken immediately to the laboratory[2] and after a careful washing in running sea water, to remove any possible outside contamination, the eggs or sperm were removed. The eggs were put into finger bowls two-thirds full of fresh sea water, care being taken in each step not to contaminate the eggs with sperm. After a sufficient number of eggs to test two or three samples of oocytin had been removed, they were gently poured into centrifuge tubes (100 cc. capacity) and allowed to settle to the bottom after which time the supernatant fluid was decanted. The tubes containing the eggs were then filled with 0.38 M strontium chloride (the sensitizing fluid), and allowed to stand 8 to 12 minutes. During this sensitization period the eggs settle to the bottom of the tubes and the supernatant fluid can be decanted. About 0.2 cc. of the thick egg suspension was transferred to each of the different finger bowls which contained the oocytin in varying dilutions[3] (1–10,000; 1–25,000; 1–50,000; 1–100,000, etc.). As the eggs are dropped into the finger bowls the latter should be gently agitated in a whirling motion to prevent them from sticking to the glass and to get the eggs thoroughly mixed in the solution. A clean medicine dropper should be placed in each finger bowl to remove samples at certain intervals for examination under the microscope. Observations were made over a period of 3 to 5 hours. The percentage of membranes formed was determined by counting the number of fertilized and unfertilized eggs in ten low power fields after thoroughly mixing the sample. Since fertilization membranes may be formed when the sea urchin eggs are kept too long in the strontium chloride solution[4] it becomes necessary to

[2] We are greatly indebted to the Director, Dr. W. K. Fisher, of the Hopkins Marine Station for the use of the laboratory and equipment to carry out these fertilization experiments.

[3] The oocytin solutions were prepared by dissolving the desired amount in 0.1 N hydrochloric acid. When the substance was in solution an equal volume of 0.1 N sodium hydroxide was slowly added and the desired dilutions were made with freshly filtered sea water.

[4] Loeb, J., Artificial parthenogenesis and fertilization, Chicago, 1913, 197.

run a control with each lot of sensitized eggs. This was done by removing the sensitized eggs from the strontium chloride to normal sea water and frequently observing to see if membranes were being formed. In every case during the course of the experiments reported in this paper the strontium chloride controls were negative. As another control eggs were mixed in a separate finger bowl with natural sperm and the number of membranes counted. All of the eggs formed fertilization membranes in less than 2 minutes showing that the eggs were mature and membrane formation was possible.

Most of the samples were prepared by the acid method which gave the best yield and the purest samples (judged from ash content and potency in causing artificial fertilization of sea urchin eggs). However, the yield is extremely variable using the same method with samples of blood from the same animal species. The results which we obtained from the preparation of a number of samples of oocytin by the different methods are summarized in Table I. From this table it is seen that oocytin is present in the blood of a large number of mammals. It seems significant that the yield of this substance from fowl blood (chicken, turkey) should so greatly exceed that obtained from any of the animals studied.

In an earlier paper[1] it was suggested that the ability of oocytin to induce membrane formation in sea urchin eggs might be due to the presence of nucleosides. This led us to try the action of the parent substance, nucleic acid, upon the sea urchin eggs. Yeast,[5] thymus,[6] and salmon sperm[6] nucleic acids were tried in the same manner and in approximately the same concentrations as were used for oocytin. With yeast nucleic acid we obtained a maximum of 9 per cent fertilization membranes, with the salmon sperm and thymus nucleic acids a maximum of 6 per cent membranes.

Having earlier found cleavage products of nucleic acid in the hydrolysate of oocytin[1] and in the present experiments finding different nucleic acids capable of inducing the formation of fertilization membranes, it would appear that nuclear material is the source of oocytin. That this nuclear material may be

[5] Obtained from Merck and Company.

[6] Prepared by Dr. C. L. A. Schmidt of this Division.

TABLE I.

*Yield of Oocytin from Various Animals by Different Methods of Preparation.*

| Date of preparation. | Source of oocytin. | Method of preparation. | Yield of oocytin per liter of serum. |
|---|---|---|---|
| | | | *mg.* |
| *1917* | | | |
| July 17 | Beef blood.* | Acid. | 44 |
| " 20 | " " * | " | 57 |
| " 26 | " " * | " | 84 |
| " 28 | " " * | " | 60 |
| *1918* | | | |
| Feb. 19 | " " * | " | 21 |
| " 20 | " " * | " | 11 |
| *1921* | | | |
| Aug. 30 | " " | $CO_2$ | 75 |
| Sept. 24 | " " † | " | 46 |
| " 24 | " " † | Neutral. | 40 |
| " 25 | " " † | Acid. | 58 |
| *1922* | | | |
| Mar. 16 | Bull " | " | 30 |
| " 16 | Ox " | " | 76 |
| " 16 | Cow " | " | 70 |
| *1921* | | | |
| Sept. 29 | Chicken blood. | $CO_2$ | 81 |
| Oct. 1 | " " | Neural. | 170 |
| " 12 | " " | Acid | 205 |
| " 17 | " " ‡ | " | 40 |
| *1922* | | | |
| Mar. 3 | " " § | " | 70 |
| *1921* | | | |
| Oct. 24 | Lamb blood. | " | 5 |
| Nov. 18 | Sheep " ¶ | " | 32 |
| *1922* | | | |
| Apr. 20 | " " | " | 60 |
| *1921* | | | |
| Aug. 30 | Pig " | $CO_2$ | 45 |
| *1922* | | | |
| Feb. 20 | " " | Acid. | 14 |
| Nov. 23 | Turkey gobbler. | " | 130 |
| " 23 | " hen. | " | 422 |
| " 27 | " gobbler. | " | 138 |
| " 27 | " hen. | " | 480 |
| Mar. 9 | Horse blood. ** | " | 90 |

TABLE I—*Concluded.*

| Date of preparation. | Source of oocytin. | Method of preparation. | Yield of oocytin per liter of serum. |
|---|---|---|---|
| *1921* | | | *mg.* |
| Sept. 3 | Horse blood ** | $CO_2$ | 62 |
| " 21 | " " ** | " | 75 |
| Oct. 28 | " " ** | Acid. | 56 |
| *1922* | | | |
| Apr. 4 | Dog " | " | 32 |

* These samples prepared by G. W. C.

† These samples were prepared simultaneously and special precautions were taken to keep conditions the same for all three samples.

‡ This lot contained mostly roosters.

§ Very young roosters, "fryers."

¶ Mostly wethers and were known to be 3 years old.

** Antipneumococcus serum from Cutter Biological Laboratories, Berkeley.

derived from the red cell nuclei is indicated by the greatly increased yield of oocytin from fowl blood which contains only nucleated red cells.

With such a variable yield of oocytin from the serum of the same animal and without a quantitative method to determine the activity of the preparation it is very difficult to state definitely whether such factors as age or sex have any influence. However, since it was possible to maintain fairly uniform conditions throughout the process of preparation and during the time of testing the activity of the substance upon sea urchin eggs, certain of our data are at least suggestive. In Table II data are presented which indicate that the oocytin prepared from old animals is more potent than that prepared from young animals of the same species.

The evidence that sex has some influence on the yield and potency of the substance is somewhat more definite. On March 16, 1922 (see Table I) we obtained samples of blood from one bull, one cow, and one ox, from which oocytin was obtained by the acid method. The yields were as follows:

Bull..... 30 mg. per liter of serum.
Ox..... 76 " " " " "
Cow..... 70 " " " " "

TABLE II.

*Effects of Age of the Animal upon the Membrane-Forming Properties of Oocytin.**

| Source of oocytin. | Membranes formed in different dilutions of oocytin.† | | | |
|---|---|---|---|---|
| | 1:10,000 | 1:25,000 | 1:50,000 | 1:100,000 |
| Sheep blood (lambs). | In 88 min., 6 per cent. | In 99 min., 7 per cent. | In 109 min., 21 per cent. | In 119 min., 19 per cent. |
| Sheep blood‡ (old wethers). | In 86 min., 23 per cent. | In 92 min., 27 per cent. | In 99 min., 31 per cent. | In 103 min., 19 per cent. |
| Chicken blood§ (young "fryers"). | In 57 min., 7 per cent. | In 64 min., 16 per cent. | In 71 min., 16 per cent. | In 68 min., 7 per cent. |
| Chicken blood. | In 50 min., 41 per cent. | In 58 min., 43 per cent. | In 65 min., 51 per cent. | In 74 min., 33 per cent. |
| Calf blood. | In 151 min., 7 per cent. | In 160 min., 15 per cent. | In 169 min., 28 per cent. | In 183 min., 16 per cent. |
| Beef " | In 158 min., 48 per cent. | In 162 min., 31 per cent. | In 188 min., 16 per cent. | In 193 min., 24 per cent. |

* The data presented in Tables II, III, and IV have been selected from a large series of experiments and in each case represents the maximum per cent of fertilization membranes formed. Due to the fact that one person (P. W. S.) made all of the counts for fertilization membranes there are variations in the time intervals (2 to 7 minutes) compared. This had no important effect upon the data presented.

† Eggs sensitized 8 to 10 minutes. Strontium chloride control negative. Sperm control gave 100 per cent of membranes in 2 to 6 minutes. Temperature 14.5°–16.5°C.

‡ Mostly wethers and known to be 3 years old.

§ Mostly young roosters, designated as "fryers."

TABLE III.

*Effects of Sex upon the Membrane-Forming Properties of Oocytin.*

| Source of oocytin. | Membranes formed in the different dilutions of oocytin.* | | | |
|---|---|---|---|---|
| | 1:10,000 | 1:25,000 | 1:50,000 | 1:100,000 |
| Cow blood. | In 95 min., 12 per cent. | In 106 min., 16 per cent. | In 115 min., 30 per cent. | In 128 min., 15 per cent. |
| Bull " | In 84 min., 48 per cent. | In 91 min., 42 per cent. | In 96 min., 58 per cent. | In 102 min., 62 per cent. |
| Ox " | In 75 min., 3 per cent. | In 81 min., 4 per cent. | In 98 min., 7 per cent. | In 105 min., 12 per cent. |

* Eggs sensitized 11 minutes. Strontium chloride control negative. Sperm contol gave 100 per cent of membranes in 2 minutes. Temperature 15°–16°C.

While the yield of oocytin from the bull blood was much less than that from the ox and cow blood it was found to be much more effective in the formation of fertilization membranes. Data showing this increased potency are presented in Table III. Two very carefully controlled collections of turkey blood[7] gave the following yields of oocytin:

November 23, 1922.
Turkey gobblers..... 130 mg. per liter of serum.
Turkey hens..... 422 " " " " "
November 27, 1922.
Turkey gobblers..... 138 mg. per liter of serum.
Turkey hens..... 480 " " " " "

TABLE IV.

*Effects of Age and Low Temperature (4°C.) upon the Membrane-Forming Properties of Oocytin.**

| Membranes formed in the different dilutions of oocytin.† | | | |
|---|---|---|---|
| 1:10,000 | 1:25,000 | 1:50,000 | 1:100,000 |
| In 20 min., 21 per cent. | In 27 min., 27 per cent. | In 33 min., 25 per cent. | In 41 min., 44 per cent. |
| In 87 min., 41 per cent. | In 94 min., 51 per cent. | In 99 min., 60 per cent. | In 105 min., 46 per cent. |
| In 144 min., 50 per cent. | In 150 min., 64 per cent. | In 157 min., 57 per cent. | In 163 min., 52 per cent. |

* The oocytin used in this experiment was prepared by the "acid method" from horse antipneumococcus serum which had been kept at 4°C. for approximately 15 months.

† Eggs sensitized 11 minutes. Strontium chloride control negative. Sperm control gave 100 per cent of membranes in 2 minutes. Temperature 15.4°C.

Since all of these fowls were of the same age, and we exercised every precaution in the collection of the blood and during the course of the preparation of the oocytin, it would seem that these results indicate that sex is a factor affecting the yield of oocytin from shed blood.

During the course of this work we were able, through the

[7] This blood was very carefully collected and separated by G. W. C. These figures are obtained from the blood of approximately 100 turkeys on each date. We are indebted to the Varsity Market, Berkeley, for their cooperation in the collection of this blood.

kindness of the Cutter Biological Laboratories, to secure a large amount of antipneumococcus serum.[8] This serum had been kept by means of tricresol and refrigeration (4°C.) for a period of approximately 15 months. Oocytin prepared from this serum by each of the three methods was very active in producing fertilization membranes. Table IV contains a brief résumé of the action of one of these samples of oocytin.

We had occasion to treat 2500 cc. of abdominal transudate (taken from a case of preagonal ascites by paracentesis abdominis) by the acid method but did not obtain sufficient material for testing.

SUMMARY.

From the work presented in this paper it is evident that:

1. Oocytin is present in the blood of the following mammals: Beef (bull, ox, cow, and calf), sheep (lamb, wether), pig, dog, horse, chicken, and turkey (gobbler and hen). It therefore seems probable that oocytin is present in the blood of all mammals.

2. Long standing of serum (approximately 15 months) at low temperatures (4°C.) has no appreciable effect upon the yield of oocytin or upon the potency of the substance in forming fertilization membranes.

3. Both the age and sex of the animal may influence the yield and membrane-forming properties of the oocytin obtained.

4. Oocytin is probably not present in appreciable amounts in the body transudates.

5. Membrane formation can be induced by thymus, salmon sperm, and yeast nucleic acids but these substances are much less effective in this respect than oocytin.

6. From the work with fowl blood it seems probable that the source of oocytin is nuclear material derived from the red cells rather than from the leucocytes as Robertson[9] suggested.

[8] The date of use had expired. Both polyvalent and univalent serum were obtained.

[9] Robertson, T. B., *Arch. Entwcklngsmechn. Organ.*, 1912–13, xxxv, 111.

# FURTHER STUDIES ON THE PARATHYROID HORMONE.

## SECOND PAPER.

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(Received for publication, July 28, 1925.)

In previous publications (1 to 4), the extraction of an active substance from the parathyroid glands of oxen, capable of influencing the calcium content of the blood of dogs, has been described. The physiological effect of this substance upon normal and parathyroidectomized animals has also been reported. In the earlier experiments aqueous extracts of the glands, prepared as outlined in one of the papers (1) referred to were used; but in the more recent work these extracts have been purified by first salting-out with sodium chloride, followed by several isoelectric precipitations. While the material thus prepared and purified was stable and caused no local reactions when given subcutaneously there were undoubtedly substances present other than the active hormone. An attempt was therefore made to isolate the physiologically active material in a pure condition and determine its chemical and physical properties. It is the purpose of this communication to report the result of this study, and to give in somewhat greater detail the method we now use for preparing active extracts.

While the character of the purified substance which we have obtained, by the procedure to be described in detail below, precludes the definite assertion that it is a pure compound, still, it behaves as a fairly well characterized individual of protein nature. The physical and chemical properties of this substance are in many ways similar to those of insulin. The material referred to above was a light gray amorphous powder very soluble in water on either side of its isoelectric point which was pH 4.8 to 4.9. At this hydrogen ion concentration, however, it was only slightly soluble. In the absence of more than very small amounts of salts,

the range of its precipitation zone was pH 4.6 to pH 5.2 with maximum flocking out at pH 4.8 to 4.9. It dissolved forming clear solutions on either side of this zone of hydrogen ion concentration. On the acid side, however, when the concentration of the acid, as for example hydrochloric acid, reached 4 per cent it was again precipitated. If much salt was present, as sodium chloride or ammonium sulfate, it would not dissolve to form a clear solution in acid; but unless the amount of salt present was large it would dissolve in alkalies. It was completely removed from acid solution by half saturation with ammonium sulfate or complete saturation with sodium chloride. It gave the common protein reactions; namely, the xanthoproteic, Millon's, biuret, ninhydrin, Hopkins-Cole, and Ehrlich's *p*-dimethylaminobenzaldehyde reactions. The Molisch carbohydrate reaction and the orcinol-HCl reaction for pentoses were negative. Sulfur and iron were present. The air-dried material contained 14.6 per cent nitrogen, but when dried in vacuum over sulfuric acid and potassium hydroxide, moisture was given up and the nitrogen content on the material thus dried was 15.5 per cent. Phosphorus was absent. It gave amorphous precipitates with picric and picrolonic acids. It was slightly soluble in absolute alcohol (0.1 per cent), quite soluble in 80 per cent alcohol, insoluble in ether, in pyridine, and in acetone. Boiling 1 hour with either 10 per cent HCl or 5 per cent NaOH destroyed its physiological activity. It was also rendered physiologically inert by the action of both pepsin and trypsin. It did not dialyze through collodion membranes to any appreciable extent. 50 cc. of a solution containing 20 units per cc. were dialyzed against distilled water in a continuous dialyzing apparatus for 12 hours. At no time during the experiment did the temperature rise above 40°C. The total dialysate, when injected into a dog, was found to be inert. It was removed from solution by Norit and Folin-Wu's tungstic acid reagent. When the material was physiologically standardized as outlined in a previous communication (3), it was found that 0.3 mg. was equivalent to 1 unit.

*Preparation of the Active Hormone.*

While a number of modifications of the original method (1) for the preparation of the hormone have been tried, the principle as outlined in the first publication gives, in our experience, the best

results when used in connection with a preliminary purification by salting-out with sodium chloride followed by several isoelectric precipitations.

The procedure for the preparation of active extracts which is now in use in this laboratory is as follows: Fresh glands are ground in a meat chopper, placed in large Pyrex tubes (5 × 45 cm.) and covered with an equal volume of 5 per cent hydrochloric acid. 3 per cent hydrochloric acid is used if the glands have been preserved in dry acetone. The tubes and their contents are then placed in a boiling water bath for from 30 minutes to 1 hour. During the digestion the mass is stirred and broken up with a glass rod until most of the material is in solution, and that which is not is quite finely divided. When this treatment is finished, the extract is diluted with four parts of hot water and set aside to cool. The fat, which separates and rises to the top of the container, congeals upon cooling and is removed mechanically. The liquid is thereupon made alkaline (pH 8 to 9) with sodium hydroxide which dissolves practically all the suspended material. Hydrochloric acid is then added with constant stirring until considerable precipitation occurs which, at the same time, permits rapid filtration. The object to be attained here is to have the liquid at a hydrogen ion concentration such that as much as possible of the active material is in solution, but at the same time rapid filtration is possible. Usually the hydrogen ion concentration is in the neighborhood of pH 5.5 to 5.6, but the proper condition can best be determined by trial. After the liquid is filtered the precipitate remaining on the filter is dissolved in weak alkali and again treated with hydrochloric acid as before. This process is repeated as long as any active material remains in the filtrates. This point is determined by noting whether upon making the filtrate acid to Congo red and saturating it with sodium chloride an appreciable amount of precipitate is formed. The active substance is separated from the filtrates by salting-out with sodium chloride, the first filtrate being worked up separately from the subsequent ones. The salting-out is done by making the solutions acid to Congo red and saturating them with sodium chloride. The substance generally flocks out and rises to the top of the liquid. The precipitate is transferred to a filter, separated from its mother liquor, dissolved in weak sodium hydroxide,

centrifuged, and the liquid adjusted to pH 4.8. The isoelectric precipitate which thus separates is either filtered or centrifuged, dissolved by the addition of hydrochloric acid, and the process repeated until the mother liquors are quite clear and devoid of color. The substance is dissolved in hydrochloric acid at a pH of about 3, Berkefelded, standardized, and is then ready for use.

The active material left in the mother liquors from the isoelectric precipitations is recovered by making the solutions acid to Congo red, saturating them with sodium chloride, filtering, and purifying the precipitates as outlined above. However, it is best to discard the first two isoelectric mother liquors from the first filtrate after acid hydrolysis as it contains much inert material which interferes with the first purification of the active substance. The subsequent mother liquors, on the other hand, are readily concentrated and purified as outlined.

In preparing the solid purified material upon which the foregoing experiments were performed, the filtrate after acid hydrolysis was purified by two precipitations with sodium chloride and ten isoelectric fractionations. After the third isoelectric precipitation the mother liquor was quite clear and almost colorless. The active substance flocked out quite sharply at the isoelectric point and required very little acid or alkali to bring it into solution again. Some of the solid was obtained at this point by suspending the precipitate, after centrifuging, in water at pH 4.8, again centrifuging, suspending the precipitate in absolute alcohol until the alcoholic concentration was 95 per cent, then adding an equal volume of dry ether, centrifuging, and washing the solid with dry ether. This substance after being thoroughly air-dried contained 14.5 per cent nitrogen. The material was then precipitated at its isoelectric point three times more, treated with water, absolute alcohol, and ether as outlined above, and dried. The product thus obtained was the preparation used for the tests enumerated above. As there noted, the nitrogen content on air-dried material was 14.6 per cent, while the material dried in vacuum over sulfuric acid, and potassium hydroxide was 15.5 per cent. Another portion of the material was reprecipitated four times more, making ten times in all, but no change in its properties could be detected.

SUMMARY.

1. A method of purification of the parathyroid hormone is described.

2. A dry amorphorus powder has been obtained which represents the purest form of the hormone yet obtained.

3. This powder contained 15.5 per cent of nitrogen and some iron and sulfur.

4. A very sharp isoelectric precipitation of the active substance was obtained at pH 4.8.

5. The purified substance was slightly soluble in absolute alcohol.

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# THE INFLUENCE OF ι-HYDROXYSTEARIC ETHYL ESTER ON ACIDOSIS.

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## INTRODUCTION.

It has long been known that the great majority of the normal fatty acids occurring in plants and animals are composed of straight chains of an even number of carbon atoms. On oxidation in severe diabetics or in normal individuals on a low carbohydrate intake, or on perfusion through the liver, they yield acetone bodies, while the odd carbon fatty acids that have been studied do not. Liver perfusion has been the method used by Embden[1] for the lower acids of the series from propionic through decoic, while feeding experiments have been resorted to for the food fats rich in oleic, palmitic, and stearic acids, and for the synthetic glyceride of margaric acid, intarvin.[2] The fatty acids from 10 to 16 carbon atoms have not been studied. The results have been quite uniform save that intarvin has yielded small amounts of acetone bodies, probably explained by the fact that the commercial product is not chemically pure.

This investigation was undertaken to determine whether or not an even carbon fatty acid with a negative group attached to the $\alpha$, $\gamma$, $\epsilon$ or some corresponding carbon atom would yield acetone on oxidation in the body. There is much evidence[3] indicating that the $\alpha$-amino acids are oxidized in the body to

[1] Dakin, H. D., Oxidations and reductions in the animal body, London, 2nd. edition, 1922, 30.

[2] Benedict, E. M., Ladd, W. S., Strauss, M. L., and West, R., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 485.

[3] Dakin, H. D.,[1] Chap. III.

α-ketonic acids which then split off $CO_2$ and undergo oxidation as a fatty acid of one less carbon atom than the original amino acid. Thus starting with α-amino stearic acid the body might itself manufacture margaric acid which is non-ketogenic. If the amino group occupied the ι, λ or some corresponding position one might expect oxidation to proceed until the acid became an α-amino acid, when the events outlined above might ensue. These are but possible steps and the fact that ι-hydroxystearic ethyl ester appears to be less ketogenic than the food fats by no means establishes this course for the reaction.

α-Amino stearic and α-amino palmitic acids have such high melting points that they are not readily absorbed from the intestine, and the keto acids were not felt to be sufficiently stable for this work. The hydroxy acids were considered but α-hydroxystearic ethyl ester and hydrogenated castor oil used as butter were not sufficiently well absorbed to yield valid results. It was finally found that ι-hydroxystearic ethyl ester, melting at 44°C., when homogenized to a cream with skimmed milk, was from 75 to 85 per cent absorbed, and this was chosen for the feeding experiments.

*Methods.*

*1. Preparation of ι-Hydroxystearic Ethyl Ester.*—ι-Hydroxystearic acid was prepared by the method of Saytzeff.[4] Concentrated sulfuric acid was added gradually to an equimolar quantity of chilled oleic acid, allowed to stand 15 to 20 hours at 15°C. or less, washed with one or two volumes of water, and the ι-sulfone stearic acid dissolved in several volumes of water. The solution was boiled until a fatty layer separated out leaving a clear underlying fluid. The fatty layer was then removed, boiled for 2 hours in alcoholic potassium hydroxide to break up lactones, and poured into a large volume of cold water acidified with sulfuric acid. The fat layer was removed in a cake when it cooled and recrystallized three times from ether. This left a yellowish white crystalline solid which on the fifth and sixth recrystallizations from ether melted at 82° and 82.5°C. Titration with alkali showed one acid group for each mol of ι-oxystearic acid. Bromine was not absorbed.

[4] Saytzeff, M., Saytzeff, C., and Saytzeff, A., *J. prakt. Chem.*, 1887, xxxv, 372.

The product after the second or third crystallization from ether was esterified with absolute ethyl alcohol in the presence of dry HCl for 6 to 8 hours on the steam bath. The reaction mass was then poured into a large volume of water and the fatty layer removed after cooling. The acetyl value of this product, which was used in the feeding experiments, was from 80-85.

Finally a weighed amount of the ester was homogenized with a known quantity of skimmed milk by the Walker Gordon Laboratories and after homogenization the total fat was determined by the Babcock method.

*Urinary Chemical Methods.*

Nitrogen was determined by Kjeldahl's method, total acetone bodies by the method of Van Slyke and Fitz,[5] total organic acids by the method of Van Slyke and Palmer,[6] and creatine and creatinine by Folin's[7] microchemical method.

The organic acid values are corrected for creatine and creatinine.

Stool fat was determined by Sharpe's method[8] over several 24 hour periods, preceded by and ending with a cathartic.

The experimental subjects were all normal.

It will be noted that the product fed was not quantitatively hydroxylated. One experiment not tabulated here, carried out before the acetyl value was determined, showed no reduction in ketosis on feeding the synthetic product. The acetyl value was then found to be only 40, so it is felt that the non-hydroxylated fraction of the ester is ketogenic.

The experiments all show a reduction in ketosis during the feeding of the ι-hydroxystearic ethyl ester and the determinations of stool fat show that the total fat absorbed during both the food fat and synthetic product periods was essentially the same. Whether the absorbed material is oxidized or stored we are unable to say. Though it was probably in part oxidized especially during the 3 day period, we have no way of establishing this point, save by feed-

[5] Van Slyke, D. D., and Fitz, R., *J. Biol. Chem.*, 1919, xxxix, 23.

[6] Van Slyke, D. D., and Palmer, W. W., *J. Biol. Chem.*, 1920, xli, 567.

[7] Folin, O., Laboratory manual of biological chemistry, with Supplement, New York, 3rd edition, 1923, 147.

[8] Sharpe, J. S., *Biochem. J.*, 1917, xi, 96.

TABLE I.

*Metabolism Experiments.*

| Subject. Date. | Weight. | Diet. | | | | Stool fat. | Fat absorbed. | Urine. | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CHO | P | Fat. Food | Fat. I-OH | | | Volume. | N | Acetone. | Org. acids in N/10 HCl. |
| **I.** | | | | | | | | | | | |
| S. ♀ *1924* | *lbs.* | | | | | *gm.* | *gm.* | *cc.* | *gm.* | *gm.* | *cc.* |
| Sept. 27–28 | 151 | 54 | 60 | 185 | 0 | | | 1350 | 8.88 | | 623 |
| " 28–29 | 151 | 54 | 60 | 185 | 0 | | | 838 | 9.52 | 0.60 | 503 |
| " 29–30 | 151 | 20 | 60 | 200 | 0 | | | 1500 | 11.35 | 0.92 | 713 |
| " 30–1 | 152.5 | 20 | 60 | 200 | 0 | | | 1380 | 10.45 | 6.44 | 1356 |
| Oct. 1–2 | 149.7 | 20 | 60 | 200 | 0 | | | 2316 | 11.00 | 7.82 | 1572 |
| " 2–3 | 148.7 | 20 | 60 | 200 | 0 | 12.7 | 187.3 | 1700 | 10.77 | 6.04 | 1382 |
| " 3–4 | 148 | 20 | 60 | 114 | 100 | 27.8 | 186.2 | 1590 | 10.90 | 3.56 | 1186 |
| " 4–5 | 148 | 20 | 60 | 200 | 0 | | | 620 | 9.58 | 9.50 | 1106 |
| **II.** | | | | | | | | | | | |
| W. ♀ *1924* | | | | | | | | | | | |
| Sept. 27–28 | 125.5 | 54 | 60 | 185 | 0 | | | 1350 | 12.60 | | 786 |
| " 28–29 | 125.0 | 54 | 60 | 185 | 0 | | | 1920 | 12.35 | 2.64 | 938 |
| " 29–30 | 124.5 | 20 | 60 | 200 | 0 | | | 1710 | 11.62 | 2.52 | 980 |
| " 30–1 | 125.0 | 20 | 60 | 200 | 0 | | | 1925 | 12.15 | 7.72 | 1538 |
| Oct. 1–2 | 123.0 | 20 | 60 | 200 | 0 | | | 2140 | 10.25 | 9.30 | 1854 |
| " 2–3 | 122.5 | 20 | 60 | 200 | 0 | 14.2 | 185.8 | 1295 | 12.10 | 7.50 | 1669 |
| " 3–4 | 122.0 | 20 | 60 | 119 | 100 | 35.6 | 178.4 | 1735 | 11.28 | 3.90 | 1220 |
| " 4–5 | 122.0 | 20 | 60 | 200 | 0 | | | 1390 | 10.87 | 5.53 | 1152 |
| **III.** | | | | | | | | | | | |
| P. ♂ *1924* | | | | | | | | | | | |
| Nov. 6–7 | 182 | 77 | 70 | 204 | 0 | | | | | | |
| " 7–8 | 181.5 | 77 | 70 | 204 | 0 | | | 2325 | 12.7 | | 535 |
| " 8–9 | | 77 | 70 | 204 | 0 | | | 2480 | 12.3 | 0.12 | 629 |
| " 9–10 | 179 | 20 | 70 | 229 | 0 | | | 1810 | 11.6 | 0.52 | 558 |
| " 10–11 | 178 | 20 | 70 | 229 | 0 | | | 2572 | 14.2 | 2.75 | 859 |
| " 11–12 | 177 | 20 | 70 | 229 | 0 | | | 2250 | 9.9 | 3.07 | 890 |
| " 12–13 | 175.5 | 20 | 70 | 229 | 0 | 6.7 | 222.3 | 3240 | 13.7 | 3.42 | 1038 |

TABLE I—*Concluded.*

| Subject. Date. | Weight. | Diet. CHO | Diet. P | Diet. Fat. Food | Diet. Fat. I-OH | Stool fat. | Fat absorbed. | Urine. Volume. | Urine. N | Urine. Acetone. | Urine. Org. acids in N/10 HCl. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| III—*Concluded.* | | | | | | | | | | | |
| P. ♂ *1924* | *lbs.* | | | | | *gm.* | *gm.* | *cc.* | *gm.* | *gm.* | *cc.* |
| Nov. 13–14 | 176 | 20 | 70 | 105 | 150 | 22.8* | 232.2* | 2500 | 12.9 | 2.02 | 1088 |
| " 14–15 | 175.5 | 20 | 70 | 105 | 150 | | | 2245 | 12.2 | 1.86 | 1099 |
| " 15–16 | 175.5 | 20 | 70 | 105 | 150 | | | 2285 | 12.5 | 1.57 | 1086 |
| " 16–17 | 175.5 | 20 | 70 | 229 | 0 | | | 2525 | 11.4 | 2.46 | 816 |
| " 17–18 | 175 | 20 | 70 | 229 | 0 | | | 2360 | 11.2 | 2.40 | 853 |
| " 18–19 | 174.5 | 20 | 70 | 229 | 0 | | | 2860 | 11.2 | 2.68 | 907 |
| " 19–20 | 174.5 | 20 | 70 | 170 | 0 | | | 2260 | 11.3 | 1.28 | 761 |

* Per 24 hours, averaged from stools of 3 days, Nov. 14 through 16.

ing experiments over longer periods, a procedure which the unpalatability of the product renders difficult. Respiratory quotients were considered of doubtful value, especially in the presence of ketosis.

There was no significant change in nitrogen excretion while feeding the ι-hydroxystearic ethyl ester, but it is interesting to note that though the excretion of acetone bodies became less, the total organic acids fell but little or remained unchanged, suggesting that the material fed was probably being oxidized to some other acid than acetoacetic and its derivatives.

ι-Hydroxystearic ethyl ester is edible but not very palatable. It is slightly laxative, the unabsorbed portion probably acting as mineral oil does. It is not nauseating and though Subject P felt definitely better while eating it, the other two subjects felt as uncomfortable as during the more severe ketosis.

## CONCLUSIONS.

ι-Hydroxystearic ethyl ester when fed to normal subjects in ketosis is followed by a reduction of the ketosis. The ester is absorbed and probably in part oxidized by the body.

We wish to thank Dr. H. D. Dakin for his friendly advice. We also wish to thank Dr. Pennock, Miss Wykoff, and Miss Sprague, who volunteered as subjects for the metabolism experiments.

# THE ANTIRACHITIC VALUE OF IRRADIATED CHOLESTEROL AND PHYTOSTEROL.

## IV. FACTORS INFLUENCING ITS BIOLOGICAL ACTIVITY.

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In previous papers we have shown that cholesterol and phytosterol can be activated, by means of ultra-violet irradiations, so as to acquire protective properties against rickets (1). It required approximately 1 mg. of the crystals, irradiated dry or suspended in water, to accomplish this result in rats which were fed a ration exceptionally low in phosphorus. Protection could be accomplished even when the irradiated cholesterol was given by the subcutaneous route, a phenomenon which gains added interest in view of the fact that cod liver oil is generally stated to be ineffective when given parenterally. A series of tests with selective filters showed that the ultra-violet radiations which render cholesterol active biologically are similar in their wave-lengths to those which were found to protect animals against rickets when they were exposed directly to the rays. This irradiated cholesterol is effective in preventing the development not only of the type of rickets brought about by a ration very low in phosphorus, but also of the type which comes about as the result of a diet low in calcium. Similar activitation could not, however, be induced in the saturated reduction products of cholesterol and phytosterol—dihydrocholesterol and dihydrophytosterol—nor in the unsaturated terpenes, cymene, and citronella oil.

It is interesting to note that some years ago Ritter (2) observed that preparations of cholesterol, prepared some 25 years previously, had become yellow in color during this interval. He writes that "the preparation had stood in glass-stoppered closed bottles

in a glass cupboard." A few years later Schulze and Winterstein (3) reported that they had kept some cholesterol for 2 years between watch-glasses, and that it had become yellow and its melting point had fallen from 146.5° to 135°. Some cholesterol, which had been stored for the same length of time in a test-tube in an atmosphere of carbon dioxide, showed a lowering in the melting point of only 1°. They concluded that the alterations were probably an oxidation effect occurring in association with light. Although these changes in cholesterol must be of a nature different from the activation which we are considering—for, as is well known, the radiations which are able to traverse glass are inert in relation to rickets—they are of interest as probably indicating that waves of visible light are also able to bring about a change in the chemical constitution of cholesterol. When cholesterol is activated by means of ultra-violet light, its melting point is but little altered, and it does not change in color. It is only after it is irradiated for long periods, for 6 or 8 hours or longer, that the melting point falls and that it begins to develop a yellow tint. Recently Steenbock and Black (4) have reported experiments on the biological activation of cholesterol. In general they have been able to confirm our experiments. Such differences as were observed may be explained by the difference in the intensity of the source of light which was employed. These investigators used the quartz mercury vapor lamp with the burner at a distance of 20 inches, using a strength of 50–60 volts, whereas we placed the burner at a distance of but 12 inches and used about 75 volts; in other words, the intensity of light employed throughout our tests was many times that employed by Steenbock and Black.

Experiments were carried out to ascertain the effect of ageing or storing on activated cholesterol, as well as on some natural foods which had been subjected to ultra-violet radiations. In previous communications we have reported that linseed and cotton seed oils maintained their acquired potency for a period of many months. Further tests have demonstrated that linseed oil which had been irradiated 1 year ago was still able to protect rats from rickets in per capita doses of 0.1 cc. a day. The results of this experiment are reproduced in Table I, where it may be noted that the inorganic phosphorus of the blood in the rats receiving activated oil was 4.06 mg., whereas it was only 1.8 mg. per cent in the

TABLE I.

*Feeding Experiments with Stored Irradiated Linseed Oil and Stored Irradiated Dry Milk.*

| Rat No. | Weight. | Rickets-producing diet. | Substance fed. | Rickets. | | | Blood P (inorganic). |
|---|---|---|---|---|---|---|---|
| | | | | Radiographic. | Macroscopic. | Microscopic. | |
| | *gm.* | | | | | | *mg. per cent* |
| 7621 | 44–45 | Low phosphorus No. 84. | 0.1 cc. linseed oil *irradiated 1 year ago* ½ hr. at 1 ft. | No. | No. | Negative | 4.06 |
| 7622 | 50–50 | | | " | " | " | |
| 7623 | 30–30 | | | " | " | " | |
| 7624 | 44–45 | | | " | " | " | |
| 7639 | 50–58 | " " | Control. | Moderate. | Moderate. | Moderate. | 1.8 |
| 7640 | 36–45 | | | " | " | " | |
| 7641 | 39–40 | | | " | " | " | |
| 7625 | 42–68 | Dry milk 5.0 | Dry milk *irradiated 3 months ago* ½ hr. at 1 ft. | No. | No. | Negative. | 5.12 |
| 7626 | 40–61 | Flour 90.0 | | " | " | " | |
| 7627 | 30–52 | Salt mixture 5.0 | | " | " | " | |
| 7628 | 34–55 | | | " | " | " | |

control rats. A similar condition holds true in regard to activated dried milk. Milk which had been dried by the roller process was tested 3 to 4 months after it had been irradiated for a period of ½ hour with the lamp at a distance of 1 foot, and was found to have lost little or none of its antirachitic potency (Table I). It will be noted that the inorganic phosphorus of the blood in these animals was within the normal range, 5.12 mg. per cent. Although natural foods, such as vegetable oils and dried milk, maintain their antirachitic potency for long periods, the same is not true of cholesterol. In a recent paper on the absorption spectra of irradiated cholesterol and phytosterol (5) it was reported that "cholesterol which had been changed in its spectral absorption by irradiation does not maintain this state." This deterioration or ageing, as gauged by spectral absorption, took place whether the cholesterol had been irradiated originally in the dried state, suspended in water, or dissolved in alcohol. Experiments were carried out to ascertain whether this ageing of cholesterol was manifest to the biological test. The cholesterol was in all cases irradiated dry in its crystalline form, and was kept for the period of the experiment, which was 28 days, either in a dry form, in water, or in oil. Table II illustrates the results of these feeding experiments. It will be noted that in all of the three series the cholesterol was unable to bring about its usual protective effect. It was most effective where it had been kept in oil throughout the experiment, and least effective where it has been kept in the dry state. As it had been shown that ½ hour of irradiation with ultra-violet light of this intensity is not the optimal amount, but that longer exposure heightens the effect, experiments were carried out with cholesterol which had been irradiated in a dry state for periods of 2 and 4 hours. The result was the same; deterioration occurred during the course of the experimental period. When the conditions of the experiment were altered somewhat and the cholesterol was irradiated suspended in water, rather than in a dry state, its antirachitic potency did not by any means diminish so rapidly. Cholesterol which had been irradiated in water for ½ hour and had been stored in water for 3 months was found to protect against rickets in daily per capita doses of 2.5 mg.

The question of the effect of the duration and intensity of irradiation is one which is associated with interesting phenomena.

TABLE II.

*Feeding Experiments with Stored Irradiated Cholesterol.*

| Rat No. | Weight. | Rickets-producing diet. | Substance fed. | Rickets. | | |
|---|---|---|---|---|---|---|
| | | | | Radiographic. | Macroscopic. | Microscopic. |
| | *gm.* | | | | | |
| 7542 | 40–49 | Low phosporus No. 84. | 0.1 cc. 1 per cent watery suspension *irradiated dry*, ½ hr. at 1 ft. *stored dry.* | Slight. | Moderate. | Slight. |
| 7543 | 36–50 | | | Moderate. | " | Moderate. |
| 7545 | 40–44 | | | " | Marked. | " |
| 7546 | 42–48 | " " | 0.1 cc. 1 per cent watery suspension *irradiated dry*, ½ hr. at 1 ft. *stored in water.* | Moderate. | " | " |
| 7549 | 40–44 | | | " | " | " |
| 7548 | 40–50 | | | Marked. | " | Marked. |
| 7547 | 44–54 | | | " | " | " |
| 7550 | 44–50 | " " | 0.1 cc. 1 per cent in linseed oil *irradiated dry* ½ hr. at 1 ft. *stored in linseed oil.* | No. | No. | Negative. |
| 7551 | 40–48 | | | Very slight. | Slight. | " (?) |
| 7553 | 34–35 | | | No (?) | " | Slight. |

It has been shown by means of the spectral absorption method, as well as by quantitative tests with a thermopile-galvanometer set, that if irradiation is prolonged for many hours cholesterol becomes less transparent, rather than more transparent, for ultraviolet rays and gives a decreased galvanometer deflection (5). The tests which have been previously reported were carried out with cholesterol dissolved in alcohol. Subsequent tests by means of the galvanometer set, with cholesterol dissolved in ether, likewise demonstrated decreased readings with cholesterol which had been irradiated for long periods. The details of these experiments will be reported at another time. Feeding experiments with cholesterol which had been irradiated for 17 hours clearly showed that the cholesterol was not active, and did not possess antirachitic properties. It should be mentioned that this cholesterol was irradiated merely at the outset and, therefore, that the factor of ageing may also have entered into this result. With this contingency in mind cholesterol was irradiated for a prolonged period every day in the dry state, and fed in 2.5 mg. amounts on the day of irradiation. These rats were fed the standard low phosphorus rickets-producing diet (Sherman-Pappenheimer), as were all the animals used in these experiments. They were not protected from rickets by the addition of the cholesterol which had been freshly irradiated for a period of 6 hours. It will be remembered that the galvanometer set showed definitely lower readings with cholesterol which had been irradiated for this length of time. Probably there is no sharp border-line between the intensity of irradiation which activates cholesterol and that which tends to its deterioration, the positive phase merging gradually into the negative phase.

Experiments were carried out also to ascertain the minimal intensity of irradiation which suffices for the activation of cholesterol. It had been ascertained previously that the subjection of linseed oil for a period of 2 minutes, using our standard intensity of light, developed antirachitic properties in this otherwise inactive vegetable oil. The rats receiving the irradiated oil had 3.42 mg. of inorganic phosphorus in their blood, whereas the control rats had but 1.83 mg. per cent. On subjecting cholesterol in water suspension to irradiation for 2 minutes, it was found insufficient, the rats developing a slight degree of rickets although they

TABLE III.

*Feeding Experiments with Cholesterol Irradiated for Short Periods.*

| Rat No. | Weight. | Rickets-producing diet. | Substance fed. | Rickets. | | | Blood P (inorganic) |
|---|---|---|---|---|---|---|---|
| | | | | Radiographic. | Macroscopic. | Microscopic. | |
| | *gm.* | | | | | | *mg. per cent* |
| 7405 | 60–70 | Low phosphorus No. 84. | 0.25 cc. 1 per cent watery suspension *irradiated 2 min.* at 1 ft. | Very slight. | Very slight. | Very slight. | 1.83 |
| 7406 | 60–60 | | | Very slight. | Very slight. | Slight. | |
| 7605 | 50–62 | " " | 0.25 cc. 1 per cent watery suspension *irradiated 15 min.* at 1 ft. | Very slight. | No. | Negative. | |
| 7606 | 40–40 | | | No. (?) | " | " | |
| 7637 | 40–52 | | | " | " | " (?) | 2.97 |
| 7638 | 40–56 | | | " (?) | " | " (?) | |
| 7617 | 46–54 | " " | Control. | Marked. | Moderate. | Marked. | |
| 7619 | 40–50 | | " | " | " | " | |
| 7620 | 40–52 | | " | " | " | " | |

received 2.5 mg. daily of this material. When the irradiation was increased to 15 minutes daily, all the rats were protected, as is shown in Table III. When, however, the cholesterol was dissolved in acetone, irradiation for 2 minutes seemed to bring about no activation whatsoever.

It is of interest to consider what is the minimal protective dose of activated cholesterol. In previous reports we have stated that 1 mg. has regularly conferred protection and has been used as the standard per capita amount in the course of these experiments. An idea of the potency of activated cholesterol in comparison with cod liver oil may be obtained from a recent statement of Dubin (6) that, on the Sherman-Pappenheimer diet, "7 mg. of fresh cod liver oil—or its equivalent in the form of the concentrate—is close to the minimal antirachitic dose." Recently Rosenheim and Webster (7) dissolved activated cholesterol in liquid paraffin and seemed to have been able to reduce the required protective amount to considerably less than 1 mg. daily. In our experience with cholesterol irradiated in liquid paraffin, 0.5 mg. was the smallest amount which protected all rats from rickets. Some experiments were also carried out to ascertain whether very large amounts of activated cholesterol were more effective than the protective doses which were being used as a routine. As much as 25 times the minimal protective dose, that is to say 0.25 cc. per capita of a 10 per cent watery suspension of cholesterol was fed. Judging by the high percentage of inorganic phosphorus in the blood—5.02 mg.—the small doses of activated cholesterol which we have been regularly using, although adequate for protection, are considerably below the optimal amount necessary to insure normal phosphorus metabolism.

A series of experiments was carried out to investigate the relationship of oxygen to the activation and activity of cholesterol. As previously reported, it was found that linseed oil which had been irradiated in an atmosphere of nitrogen was quite as potent as when it had been irradiated in air. The same procedure was followed in relation to cholesterol. The air was removed by means of suction, and nitrogen run into the tube after it had been passed through pyrogallol and over soda-lime. The quartz tube containing the cholesterol was evacuated and flushed with nitrogen several times. The cholesterol was then irradiated for ½ hour at a

distance of 6 inches, and the usual amount fed to the rats. It was found that the lack of oxygen had not interfered with activation and, as far as could be judged, that this cholesterol prevented rickets as well as that which was irradiated in air. Another type of experiment was devised to ascertain whether oxygen could be detached from the molecule of activated cholesterol and whether by this means a loss of its antirachitic properties could be brought about. Accordingly irradiated cholesterol was placed in a tube and subjected to a partial vacuum, controlled by means of a mercury manometer, for a period of 24 hours; it was then fed to the rats in the usual way. The results showed no loss in the potency of the activated cholesterol. As a corollary of this experiment, a combustion test of freshly activated and of ordinary cholesterol was undertaken. This analysis was kindly carried out by Dr. P. A. Levene, who reported that there was no apparent difference in the oxygen content between the two preparations.

With the idea that the activated cholesterol might be concentrated and thus rendered still more potent, it was recrystallized into a series of fractions. It was thought that in this way a separation might be accomplished of the material which had been activated by irradiation from that which had remained unchanged. Four fractions were prepared in the following way:

10 gm. of cholesterol were irradiated in thin layers and recrystallized five times, using 100 cc. of 95 per cent alcohol for each crystallization. It was then recrystallized five times more, using 50 cc. of 95 per cent alcohol each time. This product formed Preparation A.

The combined mother liquors were evaporated to about 100 cc. and allowed to crystallize. This product was recrystallized three times from small quantities of alcohol. This formed Preparation B.

All the mother liquors were again concentrated to 50 cc. and the product recrystallized two or three times from small quantities of alcohol. This formed Preparation C.

Finally the mother liquor was evaporated to dryness forming Preparation D. This process was carried out as quickly as possible, taking about 3 days in all. The cholesterol was irradiated dry, and the fractions kept in linseed oil throughout the period of the experiment.

Three series of tests with these four fractions were carried out. Much to our surprise the recrystallized fractions became less, rather than more potent, Fraction D being the least effective of all. Table IV reproduces the results of feeding tests with these

TABLE IV.

*Feeding Experiments with Various Fractions of Irradiated Cholesterol.*

| Rat No. | Weight. | Rickets-producing diet. | Substance fed. | Rickets. | | |
|---|---|---|---|---|---|---|
| | | | | Radiographic. | Macroscopic. | Microscopic. |
| | *gm.* | | | | | |
| 7519 | 52–60 | Low phosphorus No. 84. | Fraction A of *irradiated* cholesterol (2.5 mg.). | Moderate. | Marked. | Moderate. |
| 7518 | 60–74 | | | Marked. | " | Marked. |
| 7520 | 60–64 | | | " | " | " |
| 7521 | 50–58 | | | " | " | " |
| 7522 | 52–60 | " " | Fraction B of *irradiated* cholesterol (2.5 mg.). | " | " | " |
| 7523 | 54–59 | | | Moderate. | " | Moderate. |
| 7524 | 60–60 | | | " | " | " |
| 7525 | 60–60 | | | Slight or moderate. | " | " |
| 7527 | 60–64 | " " | Fraction C of *irradiated* cholesterol (2.5 mg.). | Marked. | " | Marked. |
| 7526 | 50–50 | | | Moderate. | " | Moderate. |
| 7528 | 50–54 | | | " | " | " |
| 7529 | 46–50 | | | " | " | " |
| 7530 | 60–56 | " " | Fraction D of *irradiated* cholesterol (2.5 mg.). | " | " | Marked. |
| 7531 | 46–49 | | | " | " | " |
| 7532 | 50–50 | | | " | " | " |
| 7533 | 54–54 | | | " | " | " |
| 7654 | 54–52 | " " | *Irradiated* cholesterol. | No. | No. | Negative. |
| 7655 | 50–50 | | | " | " | " |
| 7656 | 44–50 | | | " | " | " |
| 7657 | 50–60 | | | " | " | " |

fractions. It should be added, however, that in one of the three tests Fraction B was definitely superior to the other fractions. In this connection it may be mentioned that the cholesterol extracted from substances which contain the antirachitic principle in high degree proved to be inert. It has been found by Zucker in this laboratory and recently by Dubin and Funk (8) that cholesterol extracted from cod liver oil manifests no antirachitic potency. We extracted cholesterol from the unsaponifiable fraction of egg yolk and from beef marrow, which has been shown by Bosányi (9) to possess antirachitic power, and found this cholesterol of no value whatsoever in the protection against rickets.

It seemed of interest to ascertain whether these fractions had lost not only their biological activity but also the power of being reactivated. Accordingly, Fraction A and Fraction D were submitted to irradiation. Tests showed that the latter was not activated to an appreciable degree, and that Fractions A and C were not completely activated. It will be noted in Table V, which reproduces the data of this experiment, that Fraction A was irradiated for only 15 minutes but conferred partial protection on the rats; it was irradiated daily in water. Evidently the further purification is carried out the more we obtain of a product dissimilar, not only from activated cholesterol but from the original cholesterol itself. Another experiment devised to ascertain the possibility of reactivating cholesterol which had been deactivated, was that of irradiating some cholesterol which had been rendered inactive by excessive subjection to the ultra-violet rays; this preparation had been irradiated dry for a period of 8 hours and kept in water during the period of the experiment. It was irradiated for a second time, being exposed for ½ hour daily at a distance of 1 foot, and 2.5 mg. were fed to each rat daily. As was to be expected, activation could not be brought about again in this deactivated preparation (Table VI). Evidently, a substance quite different from the original cholesterol had been formed and the process was not reversible.

In view of the fact that various solvents were being employed in the course of the experiments with activated cholesterol, it seemed necessary to make certain that none of these had a deleterious effect upon the active principle. Accordingly a series of tests was carried out in which activated cholesterol was subjected

TABLE V.

*Attempt to Reactivate Inert Fractions of Irradiated Cholesterol.*

| Rat No. | Weight. | Rickets-producing diet. | Substance fed. | Rickets. | | |
|---|---|---|---|---|---|---|
| | | | | Radiographic. | Macroscopic. | Microscopic. |
| | *gm.* | | | | | |
| 7597 | 40–50 | Low phosphorus No. 84. | Fraction A *irradiated* 15 min. at 1 ft. | No. (?) | No. (?) | Very slight. |
| 7600 | 40–42 | | | Very slight. | " (?) | " " |
| 7598 | 60–70 | | | Slight. | " (?) | No. |
| 7601 | 40–50 | " " | Fraction D *irradiated* 15 min. at 1 ft. | Marked. | Marked. | Marked. |
| 7604 | 40–40 | | | " | " | " |
| 7602 | 44–46 | | | Moderate. | Slight. | Moderate. |
| 7603 | 60–66 | | | " | Moderate. | " |
| 7605 | 50–62 | " " | Cholesterol *irradiated* 15 min. at 1 ft. | Very slight. | No. | Negative (?) |
| 7608 | 30–30 | | | " " | " | " |
| 7606 | 40–40 | | | No. (?) | " | " |

TABLE VI.

*Attempt to Reactivate Cholesterol Which Had Been Deactivated by Prolonged Irradiation.*

| Rat No. | Weight. | Rickets-producing diet. | Substance fed. | Rickets. | | |
|---|---|---|---|---|---|---|
| | | | | Radiographic. | Macroscopic. | Microscopic. |
| | *gm.* | | | | | |
| 7595 | 40–40 | Low phosphorus No. 84. | Deactivated cholesterol. | Moderate. | Slight. | Moderate. |
| 7596 | 30–30 | | | " | Marked. | " |
| 7593 | 30–34 | " " | Deactivated cholesterol *irradiated* ½ hr. at 1 ft. | " | Moderate. | " |
| 7594 | 34–36 | | | " | " | " |

| | | | | | | |
|---|---|---|---|---|---|---|
| 7593 | 30–34 | " " | Deactivated cholesterol | " | Moderate. | " |
| 7594 | 34–36 | | *irradiated* ½ hr. at 1 ft. | " | " | " |



TABLE VII.

*Effect of Various Solvents on the Potency of Irradiated Cholesterol.*

| Rat No. | Weight. | Rickets-producing diet. | Substance fed. | Rickets. | | | Blood P (inorganic). |
|---|---|---|---|---|---|---|---|
| | | | | Radiographic. | Macroscopic. | Microscopic. | |
| | *gm.* | | | | | | *mg. per cent* |
| 7706 | 60–36 | Low phosphorus | Irradiated dry ½ hr. at 1 ft. | No. | No. | No. | |
| 7707 | 40–50 | No. 84. | In acetone 24 hrs. | " | " | " | |
| 7708 | 50–48 | | | " | " | " | |
| 7709 | 40–34 | | | " | " | " | |
| 7710 | 44–42 | " " | Irradiated dry. | Very slight. | " | Very slight. | |
| 7711 | 40–36 | | ½ hr. at 1 ft. | No. | " | No. | |
| 7712 | 58–50 | | In chloroform 24 hrs. | " | " | " | 2.31 |
| 7713 | 50–50 | | | " | " | " | |
| 7714 | 40–24 | " " | Irradiated dry | " | " | | |
| 7715 | 44–41 | | ½ hr. at 1 ft. | " | " | | 2.55 |
| 7716 | 50–50 | | In benzene 24 hrs. | " | " | No. | |
| 7717 | 40–40 | | | " | " | | |
| 7718 | 40–30 | " " | Irradiated dry | " | " | No. | |
| 7719 | 60–30 | | ½ hr. at 1 ft. | " | " | | |
| | | | In water 24 hrs. (control). | | | | |

to acetone, chloroform, and benzene. The activated cholesterol was kept in contact with these solvents for a period of 24 hours; the solvent was then removed and 2.5 mg. of the preparation were fed suspended in water. As Table VII shows, the biological activity of cholesterol was not destroyed, and probably was not lessened, by prolonged contact with any of these solvents. On the other hand, if the cholesterol is dissolved in acetone and irradiated in this solution it does not develop antirachitic potency. For example, some cholesterol dissolved in acetone was irradiated for ½ hour and then kept in a watery suspension and fed in 2.5 mg. daily amounts. The layer of the irradiated acetone solution was 8.5 mm. thick. The rats all developed rickets when this preparation was fed.

Recently Bills (10), in an article on the resistance of the antirachitic substance in cod liver oil to reagents, reported that nitrous fumes readily destroy this principle. In view of this result, the effect of nitrous fumes on activated cholesterol was ascertained. The nitrous acid was generated by the action of 20 per cent acetic acid on sodium nitrate, and the gas bubbled through a 1 per cent chloroform solution of irradiated cholesterol for 1½ hours. The solution was then evaporated to dryness and the residue taken up in sufficient water to make a 1 per cent suspension. When 0.25 cc. of this suspension was fed daily, it still protected all the rats which were being fed on the low phosphorus diet; this preliminary test is being repeated.

Another experiment undertaken to ascertain the destructive effect of agents on activated cholesterol was the subjection of preparations to a weak solution of $H_2O_2$. To 2.9 cc. of a 1 per cent watery suspension of irradiated cholesterol, 0.1 cc. of a 30 per cent $H_2O_2$ (Superoxol) was added. After contact for 1 hour, the preparation was filtered, washed, and made up to volume. Rats which were fed 2.5 mg. per capita amounts of this cholesterol in one experiment developed rickets, but in others they were protected.

In a previous communication it was reported that although human skin, as well as calfskin, possesses no antirachitic potency, either can be endowed with antirachitic properties by means of ultra-violet irradiation. Rats which were fed daily supplements of 1 gm. per capita of irradiated skin failed to develop

rachitic lesions, whereas the control animals which received non-irradiated skin regularly developed characteristic lesions. A similar experiment recently was carried out, using the sun as the source of radiation instead of the quartz mercury vapor lamp. The skin from an infant which died at the age of 12 days was exposed to sunlight for periods of 4 hours daily. This experiment was carried out with considerable difficulty on account of the variability of meteorologic conditions. It was conducted in the month of July, and during this period there were 12 days which were sunny but somewhat overcast, and only 10 days which were bright and clear. In most instances the skin was fed on the day that it was irradiated; when this was impossible, skin was given which had been exposed to sunlight on the previous day. The animals receiving this irradiated skin, in addition to the low phosphorus diet, developed rickets.

Parallel with this experiment some cholesterol suspended in water was exposed to sunlight for the same length of time. A series of rats was given 2.5 mg. of this cholesterol in addition to their rickets-producing ration. These rats also were not protected from rickets. The cause of this failure with skin and with cholesterol exposed to sunlight is to be ascribed in part to the fact that these substances could not be irradiated satisfactorily, that a sufficient intensity of light was not available.[1] This result also demonstrates the difference, from a protective point of view, between irradiating the living animal, where the activated cholesterol can be absorbed from the skin in a physiological way, and the feeding of irradiated skin. The same distinction holds true in relation to the irradiated cholesterol given by the gastrointestinal tract. It seems probable that although irradiated cholesterol of skin protects animals when given by this aphysiological route, the intensity of ultra-violet irradiation which is required to

[1] We have found recently that lettuce which was grown out-of-doors during the months of May, June, and July failed to protect rats from rickets. The lettuce was not "in head" and, therefore, well exposed to the rays of the sun; the leaves were plucked on the day of feeding and given in 10 gm. per capita amounts. The failure is to be attributed to a lack of intensity of the ultra-violet radiations of the sun. This interpretation seems warranted in view of our previous experience that lettuce can be readily and adequately activated by the rays of the quartz mercury vapor lamp.

bring about adequate activation must be far greater than where the cholesterol is activated under normal conditions.

## CONCLUSIONS.

Irradiated vegetable oil maintains its acquired antirachitic potency for at least a year and dried milk for at least 3 months. Activated cholesterol loses its potency much more readily; most rapidly when kept in the dry state, less quickly when kept in water, and slowly when stored in oil.

Prolonged intense irradiation destroys the antirachitic properties of activated cholesterol, which cannot be regained by subsequent exposure to the ultra-violet rays. The process is not reversible.

Cholesterol can be activated in an atmosphere of nitrogen. It does not lose its potency when subjected to a partial vacuum. Furthermore, no difference was found, by means of combustion, between the oxygen content of the non-irradiated cholesterol and that irradiated 1 hour.

Recrystallization of irradiated cholesterol brings about a loss of its activity, the successive fractions possessing diminishing antirachitic value. Irradiation of these deactivated fractions showed that Fraction D could not be activated; the other fractions were incompletely activated.

Activated cholesterol does not suffer a loss of potency by prolonged contact with acetone, chloroform, or benzene.

The cholesterol extracted from egg yolk, or from bone marrow, was found to have no antirachitic value.

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# CLINICAL CALORIMETRY.

## XXXIX. EXERCISE AND THE RESPIRATORY QUOTIENT IN DIABETES.

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In a previous paper (1) it has been shown, in conformity with the work of other observers, that the development of ketosis in diabetes depends upon the proportion of the foodstuffs oxidized as calculated from the respiratory quotients. It was found that ketosis could be avoided, provided that the respiratory quotient remained at or above the level of 0.76 which corresponded in theory to the oxidation of 1 molecule of glucose to each molecule of keto acid formed. It was suggested that the discrepancy between these results and those of Ladd and Palmer (2), whose patient was able to avoid ketosis though oxidizing a smaller proportion of carbohydrate, might be explained by the fact that their patient was at work, whereas ours were at rest. The present investigation was undertaken to determine the effect of exercise on the ketogenic balance, that is, on the relation between the respiratory quotient and the production of ketones. With our patients, however, it was found impractical to push the exercise to a point which would be expected to cause an increase in ketones of blood or urine beyond the limit of error of the analysis. For this reason the present paper will be confined to the effect of exercise on the respiratory quotient, leaving the question of its influence on ketosis to the literature, present and future.

### *Literature.*

Does exercise cause in the diabetic an increase in the production of ketones, and, if so, can the latter be explained by a corresponding change

in the proportion of fat oxidized? On this question the evidence is somewhat contradictory. Von Noorden (3) as cited by Forssner (5) found in diabetes no increase in the elimination of acetone as the result of exertion. Hirschfeld (4)[1] obtained the same results with normal individuals on a high fat diet. Forssner (5) made careful observations on himself when on a carbohydrate-poor diet and found a notable increase in the elimination of acetone bodies beginning immediately after exercise, and lasting for 2 or 3 days. He alone of the authors cited included beta-hydroxybutric acid, as well as acetone and acetoacetic acid in his analyses. Petri (6), in 1911, studied muscular work and its ketogenic effect on two phlorhizinized dogs and one non-diabetic individual who had been made to produce acetone by reduction of the carbohydrate of the diet. He found more acetone and acetoacetic acid in the urine on days of exercise than during the control period before or after.

On the other hand Himwich, Loebel, and Barr (7) were unable to detect any change in the concentration of ketones in the blood of diabetic patients following exercise. This is not, however, necessarily inconsistent with the work just mentioned, since the percentage in the blood shows only the excess of production over excretion.

According to the theory of the ketogenic balance an increase in ketosis would imply a depression of the respiratory quotient. Grafe and Salomon (8), in 1922, studied the quotient of a number of diabetics doing muscular work over periods of 1 hour. They found during the exercise period a consistent drop in the quotient, which they interpreted as showing a decrease in the proportion of carbohydrate oxidized. On calculating the absolute quantity of carbohydrate in the catabolism, they found a moderate increase over the rest period, but the burden of the very considerably enhanced heat production fell upon the oxidation of fat.

### *Method.*

Two non-diabetics and six diabetics were studied, and all of them except one of the controls (E. F. D. B.) remained in the metabolism ward under the same dietary control as in the previous work. They were studied in the postabsorptive state by means of the calorimeter. After a resting period of 2 hours the subject voided. Beginning early in the 3rd hour he performed exercise by stretching springs with his arms, sitting up repeatedly, marking time with his legs, turning over, or performing voluntary motions in imitation of shivering. The exercise lasted, on the average, 33 minutes and was then terminated, in order to allow the calorimeter to become stabilized for the end of the period. In the 4th hour the same procedure was repeated. This order of events was

[1] Hirschfeld (4), p. 202.

modified in three instances, indicated in Table II. The subject was rapidly removed from the calorimeter and a specimen of blood taken under oil for determination of the carbon dioxide-combining power of the plasma to compare with a similar analysis made just before the observation. No significant change was found and this was taken to mean that there was no net change in the fixed acid of the blood such as might have driven off or retained carbon dioxide and given a false value to the respiratory quotient. The sugar and in some cases the acetone bodies in the blood were also determined. The calorimeter observations were controlled by means of alcohol checks, the results of which appear in Table I. The work is based on the respiratory quotients, the accuracy of which may be judged by means of the last column. The quotient, averaged for a 3 hour period, varied from 0.667 to 0.676, as compared with the theoretical value for alcohol of 0.667. This error of the apparatus is much less than the experimental variations described below. It is conceivable, however, that there might be a change in the quotient of human beings during 4 hours even if no exercise is performed. That this change is not significant is shown by earlier work of this laboratory. Records are available from one normal individual and three diabetics, in which the average of the 3rd and 4th resting hours could be compared with the average of the 1st and 2nd. In the normal, the respiratory quotient decreased from 0.815 to 0.775. In the diabetics, the maximum variations were plus 0.042 and minus 0.003 and the average, plus 0.015.

One of the non-diabetics was a normal individual and the other can be classed as such for the purposes of this paper, although he had congenital absence of the sweat glands. The subjects have been arranged in the order of their severity, beginning with the two non-diabetics. There follow two diabetic groups of three each, the first mild and the second moderately severe. The severity was judged clinically and by means of the blood sugar. Since few of the patients had sugar in the urine at a time when they were strong enough to exercise, their carbohydrate metabolism was not taxed, and the quantity of this substance which they oxidized could not be used as a measure of severity. Only two of the patients, Jervis B. and Nicholas S., had been receiving insulin; neither of them later than the 2nd day preceding the

TABLE I.

*Alcohol Checks.*

| Date. | Hour. | Alcohol by weight. | Alcohol burned. | Heat. | | | Oxygen. | | | Carbon dioxide. | | | Water. | | | R.Q. (theory 0.667). |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Theory. | Found. | Error. | Theory. | Found. | Error. | Theory. | Found. | Error. | Theory. | Found. | Error. | |
| *1923* | | *per cent* | *gm.* | *cal.* | *cal.* | *per cent* | *gm.* | *gm.* | *per cent* | *gm.* | *gm.* | *per cent* | *gm.* | *gm.* | *per cent* | |
| Nov. 23 | 1 | 90.6 | 6.40 | 45.32 | 45.84 | +1.1 | 13.35 | 13.19 | −1.2 | 12.23 | 12.09 | −1.1 | 8.17 | 8.84 | +8.2 | 0.667 |
| | 2 | | 6.33 | 44.80 | 44.92 | +0.3 | 13.19 | 12.79 | −3.0 | 12.09 | 11.74 | −2.9 | 8.08 | 8.53 | +5.6 | 0.668 |
| | 3 | | 6.25 | 44.28 | 44.21 | −0.2 | 13.04 | 12.84 | −1.5 | 11.95 | 11.88 | −0.6 | 7.99 | 8.42 | +5.4 | 0.673 |
| | Average..... | | 6.32 | 44.80 | 44.99 | +0.4 | 13.19 | 12.94 | −1.9 | 12.09 | 11.90 | −1.6 | 8.08 | 8.60 | +6.4 | 0.669 |
| Dec. 20 | 1 | 90.6 | 8.46 | 59.91 | 59.56 | −0.6 | 17.64 | 17.31 | −1.9 | 16.17 | 15.95 | −1.4 | 10.81 | 11.50 | +6.4 | 0.670 |
| | 2 | | 8.31 | 58.86 | 59.14 | +0.5 | 17.33 | 17.24 | −0.5 | 15.89 | 15.41 | −3.0 | 10.64 | 11.05 | +3.9 | 0.650 |
| | 3 | | 8.39 | 59.38 | 60.17 | +1.3 | 17.49 | 16.90 | −3.4 | 16.03 | 15.85 | −1.1 | 10.71 | 11.22 | +4.8 | 0.682 |
| | Average..... | | 8.38 | 59.38 | 59.62 | +0.4 | 17.49 | 17.15 | −1.9 | 16.03 | 15.74 | −1.8 | 10.72 | 11.26 | +5.1 | 0.667 |
| *1924* | | | | | | | | | | | | | | | | |
| Jan. 4 | 1 | 90.6 | 6.84 | 48.45 | 47.25 | −2.5 | 14.26 | 14.77 | +3.6 | 13.08 | 13.18 | +0.8 | 8.74 | 9.30 | +6.4 | 0.649 |
| | 2 | | 6.92 | 48.97 | 48.09 | −1.8 | 14.42 | 13.62 | −5.5 | 13.22 | 12.74 | −3.6 | 8.83 | 9.03 | +2.2 | 0.680 |
| | 3 | | 6.84 | 48.45 | 47.48 | −2.0 | 14.26 | 14.07 | −1.3 | 13.08 | 13.10 | +0.2 | 8.74 | 9.13 | +4.5 | 0.677 |
| | 4 | | 6.77 | 47.92 | 49.08 | +2.4 | 14.11 | 13.71 | −2.8 | 12.94 | 12.85 | −0.7 | 8.64 | 8.99 | +4.0 | 0.682 |
| | Average..... | | 6.84 | 48.45 | 47.98 | −1.0 | 14.26 | 14.04 | −1.5 | 13.08 | 12.97 | −0.9 | 8.74 | 9.11 | +4.3 | 0.672 |
| Jan. 9 | 1 | 90.6 | 7.12 | 50.37 | 52.46 | +4.1 | 14.83 | 14.33 | −3.4 | 13.60 | 13.30 | −2.2 | 9.09 | 9.40 | +3.4 | 0.675 |
| | 2 | | 7.03 | 49.75 | 50.71 | +1.9 | 14.65 | 14.16 | −3.3 | 13.43 | 13.00 | −3.2 | 8.97 | 9.42 | +5.0 | 0.668 |
| | 3 | | 6.99 | 49.49 | 50.27 | +1.6 | 14.57 | 14.38 | −1.3 | 13.36 | 13.16 | −1.5 | 8.93 | 9.28 | +3.9 | 0.665 |
| | Average..... | | 7.04 | 49.87 | 51.15 | +2.6 | 14.68 | 14.29 | −2.7 | 13.46 | 13.15 | −2.3 | 9.00 | 9.37 | +4.1 | 0.669 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Average..... | | 7.04 | 49.87 | 51.15 | +2.6 | 14.68 | 14.29 | −2.7 | 13.46 | 13.15 | −2.3 | 9.00 | 9.37 | +4.1 | 0.669 |
| Jan. 22 | 1 | 90.6 | 7.80 | 55.22 | 54.88 | −0.6 | 16.26 | 15.62 | −3.9 | 14.90 | 14.35 | −3.7 | 9.96 | 9.75 | −2.1 | 0.668 |
| | 2 | | 7.50 | 53.13 | 53.59 | +0.9 | 15.65 | 15.28 | −2.4 | 14.34 | 14.50 | +1.1 | 9.58 | 9.67 | +0.9 | 0.690 |
| | 3 | | 7.50 | 53.13 | 53.05 | −0.2 | 15.65 | 15.75 | +0.6 | 14.34 | 14.28 | −0.4 | 9.58 | 9.76 | +1.9 | 0.659 |
| | Average..... | | 7.60 | 53.83 | 53.84 | 0.0 | 15.85 | 15.55 | −1.9 | 14.53 | 14.38 | −1.0 | 9.71 | 9.73 | +0.2 | 0.672 |
| Feb. 21 | 1 | 88.9 | 7.06 | 50.01 | 50.44 | +0.9 | 14.72 | 14.22 | −3.4 | 13.50 | 13.60 | +0.7 | 9.02 | 9.40 | +4.2 | 0.696 |
| | 2 | | 7.28 | 51.57 | 51.02 | −1.1 | 15.19 | 14.70 | −3.2 | 13.92 | 13.60 | −2.3 | 9.30 | 9.30 | ±0.0 | 0.673 |
| | 3 | | 7.06 | 50.01 | 50.45 | +0.9 | 14.72 | 14.95 | +1.6 | 13.50 | 13.58 | +0.6 | 9.02 | 9.27 | +2.8 | 0.661 |
| | Average..... | | 7.13 | 50.53 | 50.69 | +0.2 | 14.88 | 14.62 | −1.8 | 13.64 | 13.59 | −0.4 | 9.11 | 9.32 | +2.3 | 0.676 |
| Mar. 12 | 1 | 88.9 | 6.34 | 44.91 | 46.16 | +2.8 | 13.22 | 13.30 | +0.6 | 12.12 | 12.12 | ±0.0 | 8.23 | 9.16 | +11.3 | 0.663 |
| | 2 | | 6.63 | 46.97 | 46.26 | −1.5 | 13.83 | 13.23 | −4.4 | 12.68 | 12.25 | −3.4 | 8.61 | 8.75 | +1.6 | 0.673 |
| | 3 | | 6.49 | 45.94 | 46.03 | +0.2 | 13.53 | 13.24 | −2.1 | 12.40 | 12.28 | −1.0 | 8.42 | 8.65 | +2.7 | 0.675 |
| | Average..... | | 6.48 | 45.94 | 46.15 | +0.5 | 13.53 | 13.26 | −2.0 | 12.40 | 12.22 | −1.5 | 8.42 | 8.85 | +5.2 | 0.670 |
| Apr. 17 | 1 | 88.9 | 7.66 | 54.20 | 55.29 | +2.0 | 15.96 | 15.57 | −2.4 | 14.63 | 14.46 | −1.2 | 9.94 | 10.45 | +5.1 | 0.675 |
| | 2 | | 7.51 | 53.17 | 53.76 | +1.1 | 15.66 | 15.63 | −0.2 | 14.35 | 14.65 | +2.1 | 9.75 | 9.93 | +1.8 | 0.682 |
| | 3 | | 7.51 | 53.17 | 53.52 | +0.7 | 15.66 | 15.29 | −2.4 | 14.35 | 13.60 | −5.2 | 9.75 | 10.31 | +5.7 | 0.647 |
| | Average..... | | 7.56 | 53.51 | 54.19 | +1.3 | 15.76 | 15.50 | −1.7 | 14.44 | 14.24 | −1.4 | 9.81 | 10.23 | +4.3 | 0.668 |
| Total..... | | | | 1267.58 | 1273.63 | +0.5 | 373.19 | 366.09 | −1.9 | 342.10 | 337.52 | −1.3 | 229.50 | 238.51 | +3.9 | 0.670 |

observation. The former received 30 units on Mar. 25 and was observed on the 27th and 29th; the latter received 52 units on Dec. 2, and was observed on Dec. 4.

The effect on the respiratory quotient was noted, changes being tentatively assumed to reflect the proportions of foodstuffs oxidized. The quantities of protein, fat, and carbohydrate metabolized were calculated, using the table of Zuntz and Schumburg (10) as modified by Lusk (11)[1] and recently corrected by him (12). Since the patients varied greatly in their basal metabolism it was necessary to correct for this factor before making any comparisons. For this purpose the resting metabolism was arbitrarily set at 100 calories for each subject and the exercising calories increased in proportion. Both were then divided into protein, fat, and carbohydrate according to the percentage of each. These values were used to compute the averages; in so doing each patient was given a weight of one, whether one or more observations were performed with him.

The chemical methods were the same as described in Paper XXXIII (9) of this series, with the exception that after November, 1923, the Folin Wu (13) method for blood sugar was used. In determining the acetone bodies by the method of Van Slyke (14), 1 mg. of precipitate was taken as the equivalent of 1/20 mg. of acetone bodies.

*Case Histories.*

Brief summaries of the patients studied prior to December, 1923, will be found in Paper XXXIV (1) of this series. The others follow. Details concerning David L. will be found in the paper by Ladd and Palmer (2).

James D., schoolboy, born in the United States on Dec. 25, 1905, was admitted to the metabolism ward on Feb. 9, 1924. Height, 173.2 cm. Symptoms of diabetes began 2 months previously. On admission to the ward he excreted 31 gm. of glucose in less than 24 hours. The maximum excretion of ketones was 5.5 gm. on a high fat diet. He developed a tolerance for 140 gm. of carbohydrate. The highest blood sugar observed was 213 mg. per 100 cc. on Feb. 25 and the lowest carbon dioxide-combining power 57 per cent on Feb. 15.

Nicholas S., schoolboy, born in the United States in 1910, was admitted

[1] Lusk (11), p. 61.

to the metabolism ward on Nov. 5, 1923. Height, 152.6 cm. Symptoms of diabetes dated back 4 years and he had been brought out of coma three times by means of insulin. He was at no time given less than 30 units of insulin, except for two intervals, each lasting nearly 48 hours, before calorimeter observations. With 60 to 72 units a day, his tolerance for carbohydrate was only 35 gm. and the excretion of acetone bodies reached 2.3 gm. per day. The highest blood sugar observed was 345.0 gm. and the lowest carbon dioxide-combining power 57 vol. per cent.

*Results.*

The data show that in diabetes exercise is attended with a fall of the respiratory quotient below the basal level. The more important of the calorimeter data are shown in Table II, from which the severity of the exercise may be judged. The results will be found in more convenient form in Table III. Reference to Columns 10 and 11 shows that of the two non-diabetics, Chas. R. alone had a decrease in the respiratory quotient during exercise, whereas E. F. D. B. had on two occasions a change of somewhat greater magnitude in the opposite direction. The diabetic patients had without exception a fall in the respiratory quotient which was greater with the moderately severe patients than with the mild ones.

Assuming that the quotient reflects changes in the oxidation of foodstuffs, the calories have been divided into protein, fat, and carbohydrate as described in the section on method, making the basal metabolism equal to 100 calories in each case. On this basis the average increase in the carbohydrate calories oxidized as such per hour as a result of the exercise, as shown in Column 9, was 44.5 for the non-diabetics, 11.5 for the mild group, 5.0 for the moderately severe, and 8.3 for all the diabetics taken together. It is thus seen that the more severe diabetics showed almost no ability to increase their carbohydrate metabolism above the resting level. This is brought out clearly in Fig. 1, which is constructed, as in previous papers, with the total height of the column representing calories per hour, the cross-hatched portion at the bottom representing protein, the blank space at the middle, fat; and the upper vertically shaded portion, carbohydrate. To show more clearly the relation between fat and carbohydrate the base line has been set at the junction between the two, instead of at the bottom of the figure. Each pair of columns corresponds

TABLE II.

*Calorimeter Data in Terms of Averages per Hour.*

| Name. Date. Body weight. Surface.* | Time. | $CO_2$ | $O_2$ | Respiratory quotient. | Urine nitrogen. | Indirect calorimetry. | Average pulse. | Work adder.† | Relative humidity in calorimeter.‡ | Activity. | Diet in previous 24 hours. | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | Protein. | Fat. | Carbohydrate. |
| | | *gm.* | *gm.* | | *gm.* | *cal.* | | *cm.* | | | *gm.* | *gm.* | *gm.* |
| Chas. R. | 12.24 to 1.24 | | | | | | | | | | | | |
| Jan. 4, 1923. | 2.29 to 3.29 | 22.78 | 19.80 | 0.837 | 0.376§ | 66.5 | 71 | | 58 | Basal. | 80 | 121 | 255 |
| 45.7 kg. | | | | | | | | | | | | | |
| 1.46 sq. m. | 1.24 to 2.29 | 37.17 | 33.52 | 0.807 | 0.376§ | 112.1 | 81 | | 68 | Exercise. | | | |
| E. F. D.B. | 12.11 to 1.11 | | | | | | | | | | | | |
| May 10, 1922. | 2.16 to 3.16 | 23.67 | 21.17 | 0.813 | 0.386§ | 70.6 | 56 | 3 | 99 | Basal. | | | |
| 78.0 kg. | | | | | | | | | | | | | |
| 1.97 sq. m. | 1.11 to 2.16 | 51.72 | 43.51 | 0.865 | 0.386§ | 147.9 | 64 | | 100 | Exercise. | | | |
| Dec. 7, 1923. | 11.54 to 12.54 | | | | | | | | | | | | |
| 74.8 kg. | 1.34 to 2.34 | 23.39 | 21.11 | 0.806 | 0.520 | 70.1 | 55 | 17 | 69 | Basal. | | | |
| 1.92 sq. m. | 2.34 to 3.34 | 52.21 | 45.09 | 0.842 | 0.435 | 152.3 | 66 | | 85 | Exercise. | | | |
| James D. | 12.27 to 2.27 | 17.46 | 15.91 | 0.797 | 0.115 | 53.2 | 76 | 12 | 47 | Basal. | 60 | 250 | 60 |
| Feb. 15, 1924. | | | | | | | | | | | | | |
| 43.1 kg. | 2.27 to 4.27 | 24.42 | 22.38 | 0.792 | 0.291 | 74.5 | 69 | | 57 | Exercise. | | | |
| 1.48 sq. m. | | | | | | | | | | | | | |
| Ray H. | | | | | | | | | | | | | |
| Mar. 1, 1923. | 11.53 to 1.53 | 12.62 | 10.77 | 0.852 | 0.218 | 36.3 | 72 | 6 | 45 | Basal. | 70 | 200 | 70 |
| 32.3 kg. | | | | | | | | | | | | | |
| 1.23 sq. m. | 1.53 to 3.53 | 19.10 | 16.63 | 0.835 | 0.212 | 56.0 | 94 | | 43 | Exercise. | | | |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mar. 1, 1923. 32.3 kg. | 11.53 to 1.53 | 12.62 | 10.77 | 0.852 | 0.218 | 36.3 | 72 | 6 | 45 | Basal. | 70 | 200 | 70 |
| 1.23 sq. m. | 1.53 to 3.53 | 19.10 | 16.63 | 0.835 | 0.212 | 56.0 | 94 | | 43 | Exercise. | | | |
| David L. | 11.19 to 12.19 | | | | | | | | | | | | |
| Feb. 13, 1923. 54.5 kg. | 2.19 to 4.19 | 17.51 | 15.92 | 0.800 | 0.255§ | 53.0 | 54 | 12 | 67 | Basal. | | | |
| 1.61 sq. m. | 12.19 to 2.19 | 23.61 | 21.71 | 0.792 | 0.255§ | 72.3 | 61 | | 56 | Exercise. | | | |
| Jervis B.¶ | | | | | | | | | | | | | |
| Mar. 29, 1923. 56.1 kg. | 11.35 to 1.35 | 17.15 | 15.29 | 0.816 | 0.254 | 51.1 | 53 | 10 | 40 | Basal. | 70 | 100 | 70 |
| 1.68 sq. m. | 1.35 to 3.35 | 23.30 | 21.48 | 0.789 | 0.167 | 71.1 | 59 | | 49 | Exercise. | | | |
| Mar. 27, 1923.¶ 56.8 kg. | 12.14 to 2.14 | 17.48 | 16.03 | 0.793 | 0.202§ | 53.4 | 51 | 14 | 64 | Basal. | 70 | 105 | 70 |
| 1.68 sq. m. | 2.14 to 4.14 | 22.47 | 20.98 | 0.779 | 0.202§ | 69.7 | | | 60 | Exercise. | | | |
| Morris G. | | | | | | | | | | | | | |
| Feb. 16, 1923. 45.9 kg. | 11.23 to 1.23 | 13.18 | 12.42 | 0.772 | 0.218 | 41.0 | 60 | 15 | 32 | Basal. | 40 | 120 | 14 |
| 1.50 sq. m. | 1.23 to 3.23 | 22.69 | 21.81 | 0.757 | 0.254 | 72.0 | | | 45 | Exercise. | | | |
| Nich. S.¶ | | | | | | | | | | | | | |
| Dec. 4, 1923. 26.0 kg. | 12.02 to 2.02 | 11.84 | 10.76 | 0.800 | 0.236 | 35.7 | 72 | 10 | 54 | Basal. | 50 | 150 | 70 |
| 1.10 sq. m. | 2.02 to 3.02 | 18.60 | 17.58 | 0.769 | 0.189 | 58.2 | 88 | | 48 | Exercise. | | | |

* Formula of Du Bois and Du Bois.

† Does not give true readings during exercise, when the air in the chamber has to be cooled.

‡ Read at end of hour; maximum of the two periods given.

§ Average of combined urine of rest and exercise periods.

¶ Insulin therapy discontinued, last dose more than 36 hours previously.

TABLE III.

*Effect of Exercise in Diabetes.*

| Name. | Date. | When the basal metabolism is made to equal 100 calories. | | | | | | | Respiratory quotient. | | Blood. | | | | | | Urine. | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Rest. | | | Exercise. | | | | | | $CO_2$-combining power of plasma. | | Sugar. | | Acetone bodies. | | Glucose. | | Acetone bodies. | |
| | | Protein. | Fat. | Carbohydrates. | Protein. | Fat. | Carbohydrates. | Increase in carbohydrates. | Rest. | Exercise. | Before. | After. | Before. | After. | Before. | After. | Rest. | Exercise. | Rest. | Exercise. |
| | | (3) | (4) | (5) | (6) | (7) | (8) | (9) | (10) | (11) | (12) | (13) | (14) | (15) | (16) | (17) | (18) | (19) | (20) | (21) |
| | *1923* | *cal. per hr.* | *cal. per hr.* | *cal. per hr.* | *cal. per hr.* | *cal. per hr.* | *cal. per hr.* | *cal. per hr.* | | | *vol. per cent* | *vol. per cent* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *gm. per hr.* | *gm. per hr.* | *gm. per hr.* | *gm. per hr.* |
| Chas. R. | Jan. 4. | 15.0 | 44.3 | 40.7 | 15.0 | 98.6 | 55.0 | 14.3 | 0.837 | 0.807 | | | | | | | | | | |
| E. F. D. B. | May 10 | 14.5 | 52.5 | 33.0 | 14.5 | 82 8 | 112.1 | 79.1 | 0.813 | 0.865 | | | | | • | | | | | |
| " | Dec. 7 | 19.7 | 51.8 | 28.5 | 16.4 | 102.5 | 98.5 | 70.0 | 0.806 | 0.842 | | | | | | | | | | |
| " | Average | 17.1 | 52.2 | 30.8 | 15.5 | 92.6 | 105.3 | 74.6 | | | | | | | | | | | | |
| Non-diabetic. | Average | 16.1 | 48.3 | 35.7 | 15.3 | 95.6 | 80.1 | 44.5 | 0.826 | 0.839 | | | | | | | | | | |
| | *1924* | | | | | | | | | | | | | | | | | | | |
| James D. | Feb. 15 | 5.7 | 63.7 | 30.5 | 14.5 | 87.1 | 38.4 | 7.9 | 0.797 | 0.792 | 57 | 61 | 98 | 91 | 0 | 0 | 0 | 0 | 0.018 | 0.050 |
| | *1923* | | | | | | | | | | | | | | | | | | | |
| Ray H. | Mar. 1 | 15.9 | 38.0 | 46.1 | 15.5 | 73.9 | 65.0 | 18.9 | 0.852 | 0.835 | 54 | 52 | | 116 | | | 0 | 0 | 0.006 | Trace. |
| David L. | Feb. 13 | 12.8* | 58.5 | 28.8 | 12.8* | 87.2 | 36.5 | 7.7 | 0.800 | 0.792 | 61 | 60 | 111 | 109 | 4 | 7 | 0 | 0 | 0.003* | 0.003* |
| Mild diabetics. | Average | 11.5 | 53.4 | 35.1 | 14.3 | 82.7 | 46.6 | 11.5 | | | | | | | | | | | | |

| | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Jervis B. | Mar. 29 | 13.2 | 52.4 | 34.4 | 8.7 | 93.7 | 37.9 | 3.5 | 0.816 | 0.789 | | 64 | 200 | 171 | 5.3 | 7 | 0 | 0 | Trace | 0.012 |
| " | " 27 | 10.3* | 62.4 | 27.5 | 10.3* | 90.1 | 30.5 | 3.0 | 0.793 | 0.779 | 60 | 59 | 207 | 171 | 14.0 | 13 | 0 | 0 | 0.023* | 0.023* |
| " | Average | 11.8 | 57.4 | 31.0 | 9.5 | 91.9 | 34.2 | 3.3 | | | | | | | | | | | | |
| Morris G. | Feb. 16 | 14.1 | 67.5 | 18.5 | 16.4 | 133.6 | 25.5 | 7.0 | 0.772 | 0.757 | 60 | 60 | 254 | 231 | | | Trace. | Trace. | 0.027 | 0.045 |
| Nich. S. | Dec. 4 | 17.5 | 55.2 | 27.3 | 14.0 | 117.2 | 31.9 | 4.6 | 0.800 | 0.769 | 60 | 58 | 289 | 270 | 0.0 | 0 | 0.5 | | 0.025 | |
| Severe diabetics. | Average | 14.5 | 60.0 | 25.6 | 13.3 | 114.2 | 30.5 | 5.0 | | | | | | | | | | | | |
| All diabetics. | Average | 13.0 | 56.7 | 30.3 | 13.8 | 98.5 | 38.6 | 8.3 | | | | | | | | | | | | |

* Average from combined urine of rest and exercise periods.

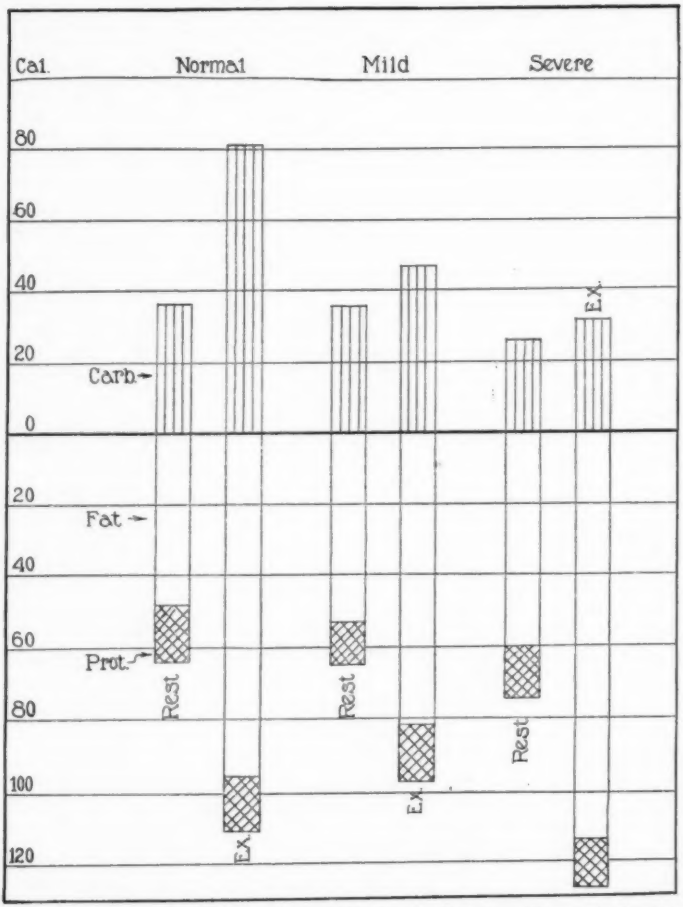


FIG. 1. Effect of exercise on the metabolism in diabetes. The entire length of the columns represents the total metabolism. The total calories at rest have been made equal to 100 and the metabolism during exercise expanded in proportion. Both have been divided into protein, fat, and carbohydrate according to the percentage present. Protein is represented by the cross-hatched portion at the bottom, fat by the blank space in the middle, and carbohydrate by the vertically shaded part of the column at the top. The base line has been set at the junction between carbohydrate and fat. The first column of each pair represents rest, and the second, exercise. The first pair was obtained from non-diabetics, the second from mild diabetics, and the third from moderately severe.

to a group of subjects. The figure shows clearly that with the more severe cases the patients perform less work with carbohydrate and more with fat, and that they have very little ability to increase the metabolism of carbohydrate above the basal level.

The chemical data of Table III are added chiefly to show the type of diabetes studied. In all the cases observed the blood sugar fell. This seems to be due to the fact that while the patients received no food in the 5 hours or so between the blood sugar determinations, oxidation of carbohydrate continued faster than it could be replaced from the stores of the body, the rate of oxidation being accelerated to a certain extent by exercise. The concentration of ketones in the blood did not change, which is to be expected in view of Himwich, Loebel, and Barr's (7) negative results with heavier exercise. The ketones of the urine tended to increase somewhat, but little importance is to be atttached to the rise.

## DISCUSSION.

Exercise has been shown above to depress the diabetic respiratory quotient in contrast to the two controls, whose quotients averaged 0.826 at rest and 0.839 during exercise. The practise of averaging the data of two individuals whose quotients were altered in opposite directions by the same procedure might be open to criticism were the results not confirmed by work from other sources. A large number of observations performed by Benedict and Cathcart (15) with a normal individual gave an average quotient during rest of 0.85 and during exercise of 0.88, showing, as with our controls, a slight increase. Neither Krogh and Lindhard (16)[3] nor Boothby and Barborka (17) found significant changes in the quotient when the basal level was intermediate, as in our controls. Our findings in conjunction with the ample literature on the subject make a satisfactory standard with which to compare the diabetics studied in this paper.

The results are in close agreement with those of Grafe and Salomon (8) who used the Jacquet type of respiratory apparatus. It remains to interpret the findings and the first question is whether they represent a true change in the proportion of foodstuffs oxidized.

[3] Krogh and Lindhard (16), p. 362.

One disturbing factor which may be excluded is a net change in the fixed acid of the blood, with driving off of carbon dioxide during exercise, or absorption during the rest period. Such changes would produce a rise or fall in the alkaline reserve as determined by the carbon dioxide-combining power of the plasma. As has been stated, this was found to be essentially the same at the end of the observations as at the beginning.

Another possible disturbing factor is the supposed conversion of fat into carbohydrate, or the reverse. A discussion of this question will be found in a paper by Loebel, Barr, Tolstoi, and Himwich (19) and by Lusk (20). At the outset it may be pointed out that such a conversion would affect the respiratory quotient only in the event that the material which resulted remained unoxidized at the end of the observation, for it is evident that the respiratory quotient is the same at the end of complete oxidation, regardless of the path by which it proceeds.

Recent studies in the physiology of muscle, as exemplified by the work of Meyerhof (22) and of Hill (21), leave little doubt that the initial phase of muscular contraction is energized by the breakdown of glycogen into lactic acid. This reaction is reversible and the greater part of the lactic acid is reconverted into glycogen. For the latter change from a lower to a higher level of potential energy, the expenditure of energy is necessary and is supplied by the oxidation of foodstuffs. It is the contention of the Hill-Meyerhof school[4] that it is the oxidation of carbohydrate alone which supplies this need, and that fat can be used only if it is changed into carbohydrate. Evidence to be detailed below makes it quite as probable, however, that fat can be used as such, without any preliminary conversion. The question is important in the interpretation of respiratory quotients, which might be profoundly affected by such a conversion, and a review of the evidence is therefore not out of place.

Krogh and Lindhard (16) put forward as a tentative hypothesis the conversion of fat into carbohydrate or the reverse on the basis of a very careful study of the respiratory quotient in normal individuals. They found that the muscular efficiency was lowered when fat was oxidized instead of carbohydrate, the waste amount-

[4] Meyerhof has restated his position on this point in a recent paper (Meyerhof, O., *Biochem. Z.*, 1925, clviii, 218.)

ing to 11 per cent of the heat of combustion of fat. This they explained as due to the energy lost in the conversion of fat into carbohydrate. They found also that quotients either very high or very low at rest tended to assume intermediate values with exercise. In this case they assumed that under basal conditions the quotient was deflected from the true value by the laying down of the material formed by conversion, and that it reached its true level only with exercise. Their third finding, the increase in the basal metabolism at extremes of the respiratory quotient, was explained as due to the energy wasted in the conversion of one form of foodstuff into the other.

Krogh's first point is in accord with the observations of Anderson and Lusk (18) with the normal dog, in which it appeared "that after glucose ingestion mechanical work may be performed at an expense of 5 per cent less energy than when no food is taken." These observations are consistent with the idea of the conversion of fat into carbohydrate, but might be explained equally well on the basis that carbohydrate has an intrinsic superiority over fat as a fuel for mammalian muscle.

The second observation, the tendency of the quotient to change from either extreme to an intermediate level with exercise, was confirmed by Boothby (17) for a normal individual, but not for a diabetic. That this point does not apply to diabetics has been shown by Grafe and Salomon (8) and by ourselves, since quotients, already low at rest, tend to fall still lower with exertion.

Meyerhof (22) found that the respiratory quotient for the excised muscle of the frog was unity, and with a similar preparation Winfield (23) found no decrease in the quantity of fat in muscle. This is evidence that under the conditions named carbohydrate alone is oxidized, but it does not follow that the same applies to living muscle *in situ*. It may signify merely that the conditions are relatively favourable for the oxidation of carbohydrate. Hill (21) states that an ample supply of the latter is available.

The results obtained with the excised muscle appear to be substantiated by the work of Hill (21) who devised a method for calculating the respiratory quotient for the extra metabolism of exercise in man. This method will be illustrated below. Hill found that the respiratory quotient was unity provided the exercise was moderate and very brief. Accepting these results, they might

mean simply that carbohydrate was more available than other substances, and not that it was the only one which could be oxidized. This applies equally well to diabetics who are well enough to exercise, since it is notoriously difficult to rid an organism of glycogen. It may be mentioned that one of Henderson and Haggard's (24) observations is not in agreement with Hill's statement, although the exercise lasted only 3 minutes. For longer exertion it seems quite certain that the respiratory quotient, as measured by this method, is not unity. Thus in the work above-mentioned, Henderson and Haggard found in athletes that the quotient of the work and recovery periods combined was approximately the same as those during rest before the work. We have used Hill's calculation with the data available from our laboratory assuming with him that the consumption of oxygen and the elimination of carbon dioxide at rest may be continued as base lines into the period of exercise. The results are shown in Table IV. The carbon dioxide eliminated solely as the result of exertion is taken as the difference between the total (Column 5) and the quantity which would have been eliminated had no work been performed (Column 4). The same subtraction is performed for the oxygen (Columns 9 and 8). Converting into liters and dividing the first remainder by the second gives the respiratory quotient of the extra metabolism due to exercise. Data were obtained in a variety of conditions, as appears in Column 12. The degree of the exertion can be judged by the quantity of oxygen consumed in excess of basal as shown in Column 10, and the duration both of the work and the subsequent period of recovery is described in Column 13. Referring to Columns 1 and 11 it will be seen that the respiratory quotient of the extra metabolism is of the same order as the basal. In the diabetics the same depression was noted that was shown in Table III. Whatever the quotient for brief exercise, that for longer periods is far from unity.

Hill lays considerable stress on the observation of a short period or element of exercise, on the ground that less disturbance would be expected in that part of the metabolism which is not concerned in the exercise. It remains to be proved, however, that the disturbance would be less in proportion to the exertion. Only in this case would any advantage accrue from the shorter periods.

The evidence for the conversion of fat into carbohydrate may be accounted for on two assumptions; first, that muscle tends to oxidize carbohydrate in preference to fat, and second, that carbohydrate is intrinsically the more efficient fuel.

On the other hand there is considerable evidence which is difficult to reconcile with the theory of the conversion of fat into carbohydrate. The observations on the phlorhizinized dog are especially important. As the result of the injection of this drug, glucose appears in the urine and increases rapidly at first, owing to the removal of carbohydrate from the body; then declines, until at the end of some days a level is reached at the rate of 3.65 gm. of glucose per gm. of urinary nitrogen. This ratio is not altered by the ingestion and consequent increase in the metabolism of fat. Once equilibrium is established, therefore, the glucose excreted is derived solely from the catabolism of protein. On the other hand various forms of carbohydrate are excreted shortly after administration. Thus Stiles and Lusk (25) have shown that glucose injected subcutaneously may be recovered quantitatively in the urine. Mandel and Lusk (26) found that there may be a complete conversion of *d*-lactic acid into glucose and elimination as such. Recently Chambers and Deuel (27, 28) have made similar observations with another object in view; they were able to recover glucose, fructose, and glycerol quantitatively, galactose to the extent of 88 per cent, and lactose 50 per cent. If so many substances of a carbohydrate nature are excreted when administered from without, it would be expected that carbohydrate, if formed internally from fat during exercise, would be eliminated also. The work of Lusk (29) shows that this is not the case. In the phlorhizinized dog, exercise which was more than sufficient to double the metabolism of fat failed to increase the elimination of glucose. The inference is that in this experiment no carbohydrate was formed from fat. In fact the very existence of a constant D:N ratio is evidence against such a theory.

This interpretation might be criticized on the ground that not all of the carbohydrate in the body is eliminated, even under full doses of phlorhizin and adrenalin; if some carbohydrate is retained, why not that which is formed from fat? It is quite true that under these circumstances, as shown by the work of Loebel, Barr, Tolstoi, and Himwich (19) muscular contraction can still be induced,

TABLE IV.

*Respiratory Quotient of the Extra Metabolism due to Muscular Exertion in Various Conditions.*

| Name. Date. | R.Q. at rest. | Duration exercise plus subsequent rest. | $CO_2$ | | | | $O_2$ | | | | R.Q. of extra metabolism due to work. | Diagnosis. | Remarks. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Eliminated at rest. | Basal for exercise and recovery (2 × 3). | Total eliminated during exercise and recovery. | Due to extra metabolism of work (5 − 4). | Consumed at rest. | Basal for exercise and recovery (7 × 2). | Total consumed during exercise and recovery. | Due to extra metabolism of work (9 − 8). | | | |
| | (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) | (9) | (10) | (11) | (12) | (13) |
| | | *hrs.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | | | |
| E. F. D. B. May 10, 1922. | 0.83 | 2.08 | 23.5 | 48.9 | 79.9 | 31.0 | 20.5 | 42.7 | 69.0 | 26.3 | 0.86 | Normal. | 1 hr. rest. Then exercise 34 min. followed by 90 min. rest. |
| E. F. D. B. Dec. 7, 1923. | 0.81 | 1.00 | 23.4 | 23.4 | 52.2 | 28.8 | 21.1 | 21.1 | 45.1 | 24.0 | 0.87 | " | 2 hrs. rest. In 3rd hr. 25 min. exercise followed by 17 min. rest. |
| Chas. R. Jan. 4, 1923. | 0.88 | 2.08 | 22.7 | 47.2 | 63.1 | 15.9 | 18.7 | 38.9 | 57.2 | 18.3 | 0.63 | Congenital absence of sweat glands. | 1 hr. rest. Then 20 min. exercise followed by 103 min. rest. |
| James D. Feb. 15, 1924. | 0.80 | 2.00 | 17.5 | 35.0 | 48.8 | 13.8 | 15.9 | 31.8 | 44.8 | 13.0 | 0.77 | Diabetes, mild. | 2 hrs. rest. In 3rd hr. 34 min. exercise followed by 20 min. rest. In 4th hr. 22 min. exercise followed by 35 min. rest. |
| Ray H. Mar. 1, 1924. | 0.85 | 2.00 | 12.6 | 25.2 | 38.2 | 13.0 | 10.8 | 21.6 | 33.3 | 11.7 | 0.81 | " " | 2 hrs. rest. In 3rd hr. exercise followed by 15 min. rest. In 4th hr. exercise followed by 21 min. rest. |
| David L. Feb. 13, 1923. | 0.80 | 4.00 | 17.1 | 68.4 | 82.6 | 14.2 | 15.7 | 62.8 | 75.5 | 12.7 | 0.81 | " " | 1 hr. rest. In each of 2 subsequent hrs. 20 min. exercise followed by 28 min. rest. Then 2 hrs. rest. |
| Jervis B. | 0.82 | 2.00 | 17.2 | 34.4 | 46.6 | 12.2 | 15.3 | 30.6 | 43.0 | 12.4 | 0.72 | " severe. | 2 hrs. rest. In 3rd hr. exercise |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Feb. 15, 1923. | | | | | | | | | | | | | lowed by 28 min. rest. Then 2 hrs. rest. |
| Jervis B. Mar. 29, 1924. | 0.82 | 2.00 | 17.2 | 34.4 | 46.6 | 12.2 | 15.3 | 30.6 | 43.0 | 12.4 | 0.72 | " severe. | 2 hrs. rest. In 3rd hr. exercise followed by rest. In 4th hr. exercise followed by 20 min. rest. |
| Jervis B. Mar. 27, 1924. | 0.80 | 2.00 | 17.5 | 35.0 | 44.9 | 9.9 | 16.0 | 32.0 | 42.0 | 10.0 | 0.72 | " " | 2 hrs. rest. In 3rd hr. exercise followed by 17 min. rest. In 4th hr. exercise followed by 18 min. rest. |
| Morris G. Feb. 16, 1923. | 0.77 | 2.00 | 13.2 | 26.4 | 45.4 | 19.0 | 12.4 | 24.8 | 43.6 | 18.8 | 0.74 | " " | 2 hrs. rest. In each of 2 subsequent hrs. exercise followed 23 min. rest. |
| Nich. S. Dec. 4, 1923. | 0.80 | 1.00 | 11.8 | 11.8 | 18.6 | 6.8 | 10.8 | 10.8 | 17.6 | 6.8 | 0.73 | " " | 2 hrs. rest. In 3rd hr. exercise followed by 24 min. rest. |
| Gerald S.* Dec. 2, 1914. | 0.76 | 2.00 | 14.4 | 28.9 | 31.9 | 3.0 | 13.9 | 27.8 | 30.4 | 2.6 | 0.84 | " | 2 hrs. rest. In 3rd hr., 10 min. exercise. In 4th hr. rest. |
| George S.† Mar. 14, 1917. | 0.78 | 2.66 | 29.3 | 77.9 | 148.7 | 70.8 | 26.4 | 70.2 | 132.1 | 61.9 | 0.83 | Malaria. | 82 min. rest. 1 min later, chill for 34 min. and recovery 125 min. |
| Joseph McC.‡ Feb. 17, 1917. | 0.82 | 2.43 | 20.9 | 50.8 | 71.5 | 20.7 | 18.6 | 45.2 | 66.4 | 21.2 | 0.71 | Typhoid vaccine. | 1 hr. rest, then from 1.15 to 3.41 p.m. period including chill which came at 1.45 to 2.15. Rest 86 min. |
| Joseph McC. Feb. 12–13, 1917. | 0.89 | 2.66 | 21.3 | 56.7 | 78.5 | 21.8 | 17.4 | 46.4 | 66.5 | 20.1 | 0.79 | " " | 2 hrs. rest, then from 10.20 to 1.00 period including chill which came at 10.21 to 10.53. Rest 127 min. |

* Allen, F. M., and Du Bois, E. F., Clinical calorimetry. Paper XVII. Metabolism and treatment in diabetes, *Arch. Int. Med.*, 1916, xvii, 1010.

† Barr, D. P., and Du Bois, E. F., Clinical calorimetry. Paper XXVIII. The metabolism in malarial fever, *Arch. Int. Med.*, 1918, xxi, 627.

‡ Barr, D. P., Cecil, R. L., and Du Bois, E. F., Clinical calorimetry. XXXII. Temperature regulation after the intravenous injection of proteose and typhoid vaccine, *Arch. Int. Med.*, 1922, xxix, 608.

and if the accepted theory holds, there must still be carbohydrate in the body to supply the reaction glycogen-lactic acid. It is evident that carbohydrate is present in the normal organism in two forms, the one more or less free, the other directly concerned in the contractile process, and resistant to excretion. If the carbohydrate supposed to be derived from fat could reach the latter category, without first passing through some stage at which it would be excreted in the urine, it might be retained. If, however, it should first take any of the forms mentioned, it would be expected to appear in the urine of the phlorhizinized dog during exercise, and this is not the case. Moreover, these forms themselves undergo transition before excretion as glucose, and it would be necessary for the hypothetical carbohydrate to avoid the additional stages as well. The evidence cited is therefore against the postulated conversion.

The above objection, whether valid or not, does not affect the observation that the phlorhizinized animal fails to oxidize carbohydrate. The fact that the substances mentioned are excreted means of course, that they are not oxidized. This has been substantiated by the study of the respiratory quotient, which remains at 0.7, the level of fat combustion, in spite of the administration of glucose (30), fructose (30), and of glycerol (28). No evidence was obtained to indicate the oxidation of carbohydrate required by the theory under discussion. It is difficult to understand how the carbohydrate supposed to originate from fat can be oxidized, and not that which is given from without. The inference is that in the phlorhizinized organism the energy required by muscular activity is furnised by fat without intermediary transformation into carbohydrate. Unless it be assumed that this drug creates an entirely new mechanism, the same applies to the normal organism, so far as the oxidation of fat is concerned.

If, then, the respiratory quotient may be taken at its face value the evidence is that the diabetic, by reason of the fundamental defect of the disease, is unable to increase the oxidation of carbohydrate to the same extent as fat. This inability was not caused by a dearth of carbohydrate. Inspection of Table III indicates that the patients who had the highest blood sugar showed with exercise the least oxidation of carbohydrate. The work with insulin has demonstrated that this sugar would have been avail-

able for oxidation if the internal secretion of the pancreas had been normal and that the failure of our patients to utilize it was an essential part of their disease.

By analogy with observations on the diabetic patient at rest, the decrease in the respiratory quotient with exercise might be expected to cause an increase in ketosis. Whether or not this is the case must be left to further investigation, perhaps along the lines of the work of Forssner (5) and of Preti (6).

## SUMMARY AND CONCLUSIONS.

1. The respiratory quotient of the exercising diabetic is depressed below the basal level.

2. Reasons are given for the belief that this depression is evidence of a decrease in the proportion of carbohydrate undergoing metabolism.

3. From a review of the available evidence the conclusion is drawn that fat can be oxidized in the mammalian organism without preliminary conversion into carbohydrate.

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# THE EFFECT OF THYROPARATHYROIDECTOMY IN DOGS UPON THE EXCRETION OF CALCIUM, PHOSPHORUS, AND MAGNESIUM.

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It is curious that, although the report by MacCallum and Voegtlin (1) that there is a lowered concentration of calcium in the blood and serum of dogs after parathyroidectomy has since been abundantly confirmed, their statement that there is an increased excretion of calcium has not been corroborated. Cooke (2), who seems to have been the only investigator to have repeated their work, found no change in the excretion of calcium after parathyroidectomy, but did observe an increase in the excretion of magnesium.

In spite of this contradictory report, the statement of MacCallum and Voegtlin has been almost universally accepted. The decreased concentration of calcium in the blood seems to have been regarded as proof that there must be an antecedent increased excretion.

Salvesen (3, 4) found that calcium injected as the chloride into dogs in tetany in such quantities as was calculated to restore the calcium content of the plasma and lymph to its normal value was promptly excreted through the intestine and drew the following conclusion.[1]

"It is apparent from the experiments that in parathyroidectomized dogs there is a lowered threshold for the excretion of calcium in the intestines and this is apparently the cause of the calcium deficiency and thereby of all the symptoms."

If this lowered threshold be real, it is difficult to understand why the calcium of the bones is not drawn upon to a great extent

[1] Salvesen (3), p. 455.

after parathyroidectomy, giving rise to an increased excretion through the urine and feces that would be absolutely unmistakable. The significance of the experiments of Salvesen will be discussed in a subsequent publication. Suffice it now to point out that severe losses of calcium may occur in acidosis, pregnancy, and lactation, particularly on diets poor in calcium, without leading to tetany or even to an appreciable lowering of the concentration of calcium in the blood. Why is the calcium of the bones not available for the maintenance of the calcium content of the blood after parathyroidectomy just as it is in these other conditions?

The question seems to be of sufficient importance to warrant detailed examination of the published results of MacCallum and Voegtlin. Such examination leads to grave doubt as to the significance of these results as indicating an increased excretion of calcium.

Their Dog 1807[2] received 800 cc. of milk daily for 5 days. On the last three of these, the ratios of Ca $\times$ 1000: N were 16.7, 5.20, and 6.25. After parathyroidectomy, the dog received 800 cc. of water daily. The ratios of Ca $\times$ 1000:N on the next 4 days were 4.35, 5.46, 5.00, and 6.03. Certainly, there is here no evidence of any marked increase in the excretion of calcium. No feces were obtained. The dog developed tetany on the 2nd day after the operation and died on the 4th day.

In the next experiment[3] the excretion of calcium in the urine shows absolutely no increase. The feces were analyzed but none were obtained after the successful second operation (the first operation was not followed by tetany).

Another dog weighing 19.2 kilos,[3] received 500 cc. of water daily, beginning March 13. The collection of urine was begun on March 15. The excretion of nitrogen in the urine up to, but not including, March 21, the day of the operation, averaged 4.54 gm. per day, and that of calcium, 0.026 gm. The ratio of Ca $\times$ 1000:N was 5.72. Tetany appeared on March 24. The average N excretion for the 3 days between the operation and the appearance of tetany was 3.79 gm. per day; that of Ca, 0.023 gm. The ratio of Ca $\times$ 1000 : N was 6.07. After tetany appeared, the amount of nitrogen in the urine first rose, then fell. The average

[2] MacCallum and Voegtlin (1), p. 144.
[3] MacCallum and Voegtlin (1), p. 147.

of the 6 days was 7.37 gm., that of calcium was 0.029 gm., and the ratio was 3.94. The first feces recorded were passed on March 19. They contained 0.127 gm. calcium. On the following day feces containing 0.003 gm. calcium were excreted and on the day thereafter (the day of the operation), the feces contained 0.153 gm. calcium. Perhaps the material in this last sample of feces was actually excreted by the intestine on this day but it seems to the writer more probable tht the colon merely emptied itself, possibly in the struggle incident to etherization of material that had been excreted into the intestine on the previous day, or even earlier. The next feces were obtained on the 8th day after the operation and contained 1.368 gm. calcium, an average of 0.171 gm. per day. This is not so greatly different from the amount passed on the day of the operation. It is, however, far greater than the amount passed in the 6 days before operation. But whether or not it represents a really increased excretion, it must not be forgotten that these feces were not passed until after 6 days of tetany. Any increased excretion of calcium may have been the effect of the tetany rather than its cause.

In the last experiment,[4] the calcium excreted in the urine before the unsuccessful first operation averaged 0.016 gm. per day. From then to the second, successful operation, the average excretion was 0.014 gm. calcium per day. During the next 11 days, during the last 9 of which the dog was in tetany, it varied between 0.011 and 0.019 gm., except on the 3rd day (1st day of tetany) when the value of 0.092 gm. was recorded. This is so strikingly different from anything observed in the urines of the other parathyroidectomized dogs as to suggest that there may have been some error. No feces were obtained after the successful operation.

Experiments such as these cannot be accepted as evidence of an increased excretion of calcium after parathyroidectomy, when such increased excretion could not be confirmed by Cooke. Salvesen[5] states: "Although Cooke failed to demonstrate the increased calcium excretion following parathyroidectomy, this is no proof that it does not take place, as there are many sources of error in experiments of this kind, especially when the colon is not washed out."

[4] MacCallum and Voegtlin (1), p. 148.
[5] Salvesen (4), p. 136.

It is quite true that there may have been errors due to retention of fecal matter in the colon in Cooke's experiments but they were as likely to have occurred in the one experiment by MacCallum and Voegtlin in which feces were obtained after a successful parathyroidectomy.

However, careful examination of Cooke's experiments shows that these are as little to be accepted as evidence of an unchanged excretion of calcium after parathyroidectomy as those of MacCallum and Voegtlin to be accepted as indicating an increase.

For instance, in Table IV,[6] the excretion of calcium in the urine during the 5 days before the operation was 3.9, 6.4, 4.0, 4.5, and 6.0 mg., respectively. On the following days it was 4.4, 4.3, 3.1, 3.8, 4.4, 4.9, 3.9 mg., etc. The feces of 4 days of the control period, on the first 2 of which the dog received the standard diet of beef heart, cracker meal, and lard, contained 0.1857 gm. Ca, or 46.4 mg. per day. The feces of the day of the operation and the day *preceding* contained 50 mg. or 25 mg. per day. On the following day, the feces contained 10.2 mg. Ca and, on the 3rd day after that, 119.7 mg. or 39.9 mg. per day. After the next 5 days, on the 2nd and 3rd of which the dog received the standard food, the feces contained 435.1 mg. Ca, or 145 mg. per day.

In the experiment summarized in Cooke's Table VI[7] the dog was fed up to 3 days before the operation. The excretion of calcium in the urine on these 3 days was 3.8 mg. per day. Thereafter, it varied between 2.0 and 6.9 mg. the average for 7 days being 4.9 mg. The dog defecated on the 2nd day before the operation and again on the day immediately preceding. The calcium in the second specimen of feces amounted to 0.1324 gm. No feces were passed until the 7th day after the operation when feces containing 0.2964 gm. calcium were voided. This was the equivalent of 0.0423 gm. per day. But this decrease is scarcely greater than was observed in a fasting experiment upon the same dog some days previously.[7] In that experiment, the fecal excretion of calcium diminished from an average of 0.85 gm. per day during the period of feeding to an average of 0.067 gm. per day during the 6 days of starvation. A return to the standard diet increased this to 0.3 or 0.4 gm. calcium per day.

[6] Cooke (2), p. 50.
[7] Cooke (2), p. 51.

This was followed by the previously described deprivation of food and operation.

In the remaining experiment,[8] the dog died on the day after the operation and no feces were obtained. The excretion of calcium in the urine was not affected.

It is obvious that data such as these are entirely inconclusive. Since removal of the parathyroids is unquestionably followed by a disturbance in calcium metabolism, it seemed desirable to determine whether or not there really is an increased excretion of calcium. Accordingly, a series of metabolism experiments was carried out. These were quite conclusive in showing that there is, after thyroparathyroidectomy in dogs, no increase in the excretion of calcium.

Moreover (5), we have found that the injection of a potent parathyroid extract, which was found by Collip (6) to increase the concentration of calcium in the blood, was followed by an increase in the excretion of both phosphorus and calcium. The relation of the parathyroids to calcium excretion is, evidently, exactly the reverse of what has previously been believed.

Equally as curious as the failure to find in an increased excretion of calcium the cause of the diminished concentration in the blood is the failure to find an increased concentration of phosphate in the blood as a result of the retention of phosphate that is the most striking change in metabolism after parathyroidectomy (7). Apparently, the phosphate and calcium are deposited in the tissues at the same time and, presumably, together.

## EXPERIMENTAL.

In order to obtain accurate results, it was essential to have a diet which could be kept constant throughout the experiment. Since, after the operation, the dogs frequently refuse food, it was necessary to have the food of such consistency that it could readily be given by stomach tube. In order to obtain feces fairly regularly, without resorting to such a troublesome, and possibly disturbing, procedure as colonic irrigation, it was essential to add some bulky inert material.

The diets used consisted of dried beef heart, cracker meal, lard

[8] Cooke (2), p. 52.

or maize oil, infusorial earth, and water. Because of its use in the serum diagnosis of syphilis, dried beef heart is now a regular article of commerce.[9] The dogs were first fed mixtures containing fresh beef heart and then, on successive days, one-third or one-fourth of the fresh beef heart was replaced by an equivalent amount of the dried product. In that manner, the dogs learned to eat this mixture very promptly and, after several days on this mixture, the collection of the excreta was begun. The addition of the infusorial earth secured defecation almost every day. The feces were firm, dried rapidly, and could be powdered very easily.

The dogs were not catheterized and the periods overlap somewhat. Since the amount of feces voided on the different days varied greatly and since the bulk of the feces consisted of $SiO_2$, it has been thought best to report not only the total amounts excreted but also the amount of phosphorus, calcium, and magnesium per gm. of $SiO_2$. Changes in the ratio of excretion would be promptly indicated by a change in these ratios.

All analyses were made in duplicate.

The following analytical procedures were used: nitrogen, Kjehldahl; inorganic phosphate, Brigg's modification of the Bell-Doisy method (8); total phosphorus, calcium, and magnesium, the specimens were oxidized with sulfuric and nitric acids. After diluting, the silica (in the case of the feces) was filtered out, washed, ignited, and w eighed. The filtrate, or the diluted oxidation mixture, was made alkaline with $NH_4OH$ and then acid with acetic acid. Ferric chloride was then added until a permanent red color was produced, the mixture was boiled, a few drops of *pure*, dilute $H_2O_2$ were added and the precipitate was filtered out and washed with boiling water. The precipitate was dissolved in dilute $HNO_3$ and an aliquot was taken for the determination of phosphorus by precipitation with molybdate and titration, using HCHO to bind the ammonia (9).

The filtrate from the iron precipitate was evaporated, any additional precipitate that separated being added to that already obtained, and then precipitated with ammonium oxalate. In the course of this work it was found that more rapid and more complete precipitation of the calcium was obtained, when only a little

[9] We are indebted to Lehn and Fink for a generous supply of dried beef heart.

was present, if a small quantity of the evaporated liquid was added to the ammonium oxalate solution, the mixture stirred until crystals appeared, and then added to the remainder of the liquid to be precipitated. After standing 24 hours, the calcium oxalate was filtered on a Gooch, washed, and titrated with $KMnO_4$. A 0.1 N solution was used for the feces and a 0.01 N solution for the urines.

The filtrate from the calcium oxalate was treated with $Na_2HPO_4$ and $NH_4OH$. The precipitated $MgNH_4PO_4 \cdot 6H_2O$ was filtered on a Gooch, dried at room temperature over $CaCl_2$, and weighed. The infusorial earth used in the third experiment appears to have been more soluble than that used in the first two. In this experiment, the ammonium magnesium phosphate was contaminated with enough silicate to make filtration on a Gooch very difficult. These precipitates were filtered on paper, washed, and then dissolved in nitric acid. Phosphorus was then determined by molybdate precipitation and titration and the magnesium calculated.

*Experiment 1* (*Tables I and II*).—The dog developed a hematoma at the site of the operation. Apparently, this was responsible for the increase in the excretion of nitrogen. The increased protein metabolism which this represented was, presumably, accompanied by an increased liberation of phosphoric acid. This seems to have masked the fall in phosphate excretion which has otherwise been uniformly observed after a successful parathyroidectomy. Although the total amount of phosphorus did not diminish, the ratio of phosphorus to nitrogen fell in the usual manner.

In an attempt to secure accurate delimitation of the feces belonging to each period, 0.2 gm. carminic acid was added to the first feeding of each period. This did not appear in the feces until at least 24 hours later and elimination was generally not completed until 36 or 48 hours after feeding. If the last day of the fore period had been immediately followed by the operation, the feces derived from the last feeding of the fore period might have been affected by changes in metabolism due to the operation. Consequently, the fore period was closed 24 hours before the operation.

As is quite obvious from the figures given in the table, the excretion of calcium was not only not increased after the operation but was actually decreased. The decrease became even more marked

TABLE I.

Dog. 27. Weight 8.2 kilos. Fed daily a mixture of 20 gm. dried beef heart, 20 gm. cracker meal, 20 gm. lard, 12 gm. infusorial earth, and 425 cc. $H_2O$.

| Date. | Urine. | | | | | Feces. | | | | | | | | Remarks. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | Inorganic P. | Total P. | Ca | Mg | Weight. | $SiO_2$ | P | Ca | Mg | Per gm. $SiO_2$ | | | |
| | | | | | | | | | | | P | Ca | Mg | |
| *1924* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | |
| Sept. | | | | | | | | | | | | | | |
| 23 | 1.405 | 0.093 | 0.089 | 0.0003 | 0.0050 | | | | | | | | | |
| 24 | 4.11 | 0.323 | 0.340 | 0.0077 | 0.0253 | | | | | | | | | |
| 25 | 3.89 | 0.311 | 0.321 | 0.0047 | 0.0234 | | | | | | | | | |
| 26 | 4.06 | 0.320 | 0.321 | 0.0035 | 0.0247 | 60.5 | 41.2 | 0.153 | 0.290 | 0.1320 | 0.00370 | 0.00704 | 0.00322 | |
| 27 | 4.92 | 0.375 | 0.302 | 0.0073 | 0.0257 | | | | | | | | | |
| 28 | 4.09 | 0.330 | 0.312 | 0.0001 | 0.0251 | | | | | | | | | |
| 29 | 3.90 | 0 276 | 0.288 | 0.0001 | 0.0246 | 39.0 | 24.1 | 0.129 | 0.232 | 0.0814 | 0.00534 | 0.00965 | 0.00337 | |
| 30 | 3.10 | 0.243 | 0.236 | 0.0001 | 0.0224 | | | | | | | | | |
| Oct. | | | | | | | | | | | | | | |
| 1 | 5.96 | 0.328 | 0.334 | 0.0050 | 0.0275 | | | | | | | | | Removed thyroids and parathyroids, Oct. 1, 9.45 a.m. |
| 2 | 6.86 | 0.269 | 0.296 | 0.0006 | 0.0256 | 37.8 | 25.8 | 0.112 | 0.185 | 0.0780 | 0.00435 | 0.00716 | 0.00302 | |
| 3 | 6.44 | 0.413 | 0.488 | 0.0001 | 0.0429 | | | | | | | | | Twitching all day. |
| 4 | 4.11 | 0.227 | 0.267 | 0.0001 | 0.0368 | | | | | | | | | " " " |
| 5 | 13.11 | 0.642 | 0.796 | 0.0001 | 0.0828 | | | | | | | | | " " " Urine contaminated with blood from wound. |
| 6* | 2.09 | 0.100 | 0.129 | 0.0001 | 0.0194 | 45.8 | 30.3 | 0.112 | 0.169 | 0.0902 | 0.00370 | 0.00558 | 0.00298 | Died between 6 and 9 p.m. in severe tetany. Feces expressed from colon. |

* About 12 hours.

after tetany appeared. These changes are even more apparent when the excretion of calcium per gm. of silica is considered.

Since the amount of infusorial earth fed was a constant, the amount of silica in the feces, per day, should have been the same in the different periods. This was not the case. Obviously, the delimitation of the periods was not accurate.

The total amount of silica voided in the 13.5 days of the experiment was 121.4 gm. Assuming that the errors involved in delimitation at the beginning and end of the experiment are negligible when spread out over the entire 13.5 days, the daily average excretion of silica was 9.02 gm. Multiplying this by the number of days in each period and by the amount of phosphorus, calcium, and magnesium per gm. of silica voided in each period, we obtain the

TABLE II.

*Difference between Observed and Calculated Fecal Excretion of Phosphorus, Calcium, and Magnesium in the Several Periods of Experiment 1 (See Text).*

| Period. | Calculated. | | | Found. | | | Excess of found over calculated. | | |
|---|---|---|---|---|---|---|---|---|---|
| | P | Ca | Mg | P | Ca | Mg | P | Ca | Mg |
| | gm. | gm. | gm. | gm. | gm. | gm. | gm. | gm. | gm. |
| I | 0.133 | 0.253 | 0.1160 | 0.153 | 0.290 | 0.1320 | +0.020 | +0.037 | +0.0160 |
| II | 0.144 | 0.261 | 0.0910 | 0.129 | 0.232 | 0.0814 | −0.015 | −0.019 | −0.0096 |
| III | 0.117 | 0.193 | 0.0812 | 0.112 | 0.185 | 0.0780 | −0.005 | −0.008 | −0.0032 |
| IV | 0.117 | 0.176 | 0.0939 | 0.112 | 0.169 | 0.0902 | −0.005 | −0.007 | −0.0037 |

values given in the first part of Table II. The figures in the following three columns of the table show the differences between these values and those obtained directly. They represent the maximum errors due to faulty delimitation of the periods. Since they are much smaller than the changes observed, they leave no doubt as to the reality of the decrease in the excretion of calcium.

*Experiment 2 (Table III).*—The excretion of phosphorus in the urine showed the usual fall after the operation. A large part of the urine of the day of the operation was lost. The remainder was used for the determination of phosphorus, calcium, and magnesium. None could be spared for the determination of nitrogen or inorganic phosphate.

Due to a misunderstanding, the silica obtained in the analyses of

TABLE III.

Dog 28. Weight 7.9 kilos. Fed daily a mixture of 10 gm. dried beef heart, 30 gm. cracker meal, 30 gm. lard, 12 gm. infusorial earth, and 300 cc. $H_2O$.

| Date. | Urine. | | | | | Feces. | | | | | | | | Remarks. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | Inorganic P. | Total P. | Ca | Mg | Weight. | P | Ca | Mg | Total ash. | Per gm. ash. P | Per gm. ash. Ca | Per gm. ash. Mg | |
| *1924* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | |
| Nov. | | | | | | | | | | | | | | |
| 11 | 1.36 | 0.108 | 0.109 | 0.0001 | 0.0138 | 16.9 | 0.0360 | 0.0851 | 0.0135 | 13.6 | 0.00265 | 0.00626 | 0.0010 | |
| 12 | 1.39 | 0.101 | 0.102 | 0.0001 | 0.0150 | 11.1 | 0.0248 | 0.0606 | 0.0111 | 8.9 | 0.00278 | 0.00681 | 0.0013 | |
| 13 | 1.37 | 0.091 | 0.092 | 0.0001 | 0.0110 | 14.2 | 0.0308 | 0.0746 | 0.0142 | 11.1 | 0.00278 | 0.00678 | 0.0013 | |
| 14 | 1.13 | 0.075 | 0.070 | 0.0001 | 0.0078 | 16.0 | 0 0360 | 0.0668 | 0.0160 | 12.9 | 0.00280 | 0.00519 | 0.0013 | |
| Average.. | 1.31 | 0.094 | 0.093 | 0.0001 | 0.0119 | 14.55 | 0.0319 | 0.0718 | 0.0137 | 11.6 | 0.00275 | 0.00519 | 0.0118 | |
| 15 | Lost | | 0.018 | 0.0001 | 0.0171 | 15.2 | 0.0360 | 0.0704 | 0.0167 | 11.4 | 0.00315 | 0.00615 | 0.0015 | Thyroparathyroidectomy. |
| 16 | 1.97 | 0.029 | 0.035 | 0.0001 | 0.0219 | 13.2 | 0.0261 | 0.0504 | 0.0132 | 9.1 | 0.00282 | 0.00553 | 0.0015 | |
| 17 | 1.54 | 0.031 | 0.033 | 0.0001 | 0.0072 | 25.0 | 0.0430 | 0.0845 | 0.0250 | 17.3 | 0.00242 | 0.00476 | 0.0014 | Twitching all day. |
| Average.. | 1.76 | 0.030 | 0.029 | 0.0001 | 0.0154 | 17.8 | 0.0350 | 0.0684 | 0.0183 | 12.6 | 0.00278 | 0.00543 | 0.00145 | |
| 18 | | | 0.280 | 0.0081 | 0.0157 | 23.0 | 0.0658 | 0.0504 | 0.0207 | 7.14 | 0.00923 | 0.00719 | 0.0029 | Dog killed at end of period. Urine contaminated with vomit and feces. Large intestine contained dark red (bloody) material. This was analyzed and reported as feces. |

the feces was not weighed. There was not sufficient material to permit of a repetition but there was enough material to secure satisfactory determinations of the total ash, of which the silica constituted about 90 per cent.

The feces passed on the day of the operation were probably formed on the previous day. The amount of calcium per gm. of ash was just a little less than the average for the fore period. It became progressively less on each of the 2 following days. The dark, bloody mass found in the intestine after the death of the dog contained more calcium per gm. of ash but, quite obviously, this was due to the admixture of blood and cannot be considered as the cause of the preexistent tetany.

*Experiment 3 (Table IV).*—After the operation, the phosphorus excretion in the urine fell in the manner that is characteristic of a successful parathyroidectomy. A faint, and somewhat doubtful, tremor was observed on the 2nd, 3rd, and 4th day after the operation but no obvious tetany developed.

On the 5th day, the phosphorus excretion in the urine had returned to normal level, although, because of the increase in nitrogen excretion, the ratio of phosphorus to nitrogen was still low. Only one other dog out of more than fifty that have shown this drop in phosphorus excretion has failed to develop tetany. It seemed that the animal would soon recover from the parathyroid insufficiency. Accordingly, he was bled to death. The serum from the first blood obtained contained 7.63 mg. calcium per 100 cc. Evidently, there was a parathyroid insufficiency but this was not quite sufficient to produce tetany. Perhaps the fact that the dog was rather old was of some significance.

In the two preceding experiments, the calcium excretion in the urine had been negligible but, in this experiment, it was, before the operation, considerable, the average being 34.2 mg. per day. After the operation it promptly declined to 0.4 or 0.5 mg. per day.

Because of the irregularities in defecation, the data for the feces, unless carefully analyzed, are apt to be rather confusing. The operation was performed at 10.20 a.m. The animal was fed at about 1 p.m. and defecated between that time and 3 p.m. Under ordinary conditions, these feces would in all probability have been similarly voided shortly after feeding and would, consequently have been included with those voided during the previous day and

TABLE IV.

Dog 33. Weight 16.2 kilos. Fed daily a mixture of 42.5 gm. dried beef heart, 57 gm. cracker meal, 70 cc. maize oil, 22 gm. infusorial earth, and 925 cc. $H_2O$.

| Date. | Urine. | | | | | Feces. | | | | | | | | Remarks. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | Inorganic P. | Total P. | Ca | Mg | Weight. | P | Ca | Mg | $SiO_2$ | Per gm. $SiO_2$ | | | |
| | | | | | | | | | | | P | Ca | Mg | |
| *1925* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | |
| Apr. | | | | | | | | | | | | | | |
| 2 | 6.71 | 0.461 | 0.473 | 0.0531 | 0.028 | 61.3 | 0.347 | 0.610 | 0.128 | 38.0 | 0.00914 | 0.0160 | 0.00338 | |
| 3 | 4.56 | 0.167 | 0.174 | 0.0283 | 0.014 | | | | | | | | | |
| 4 | 5.95 | 0.398 | 0.383 | 0.0394 | 0.012 | 27.6 | 0.179 | 0.248 | 0.061 | 19.0 | 0.00941 | 0.0131 | 0.00321 | |
| 5 | 5.29 | 0.296 | 0.299 | 0.0249 | 0.027 | | | | | | | | | |
| 6 | 5.31 | 0.348 | 0.328 | 0.0095 | 0.036 | 28.4 | 0.176 | 0.261 | 0.104 | 17.6 | 0.01003 | 0.0149 | 0.00590 | |
| 7 | 7.95 | 0.616 | 0.650 | 0.0465 | 0.055 | 56.8 | 0.340 | 0.546 | 0.169 | 36.4 | 0.00934 | 0.0152 | 0.00464 | |
| 8 | 3.43 | 0.127 | 0.134 | 0.0123 | 0.014 | 23.6 | 0.156 | 0.227 | 0.064 | 15.1 | 0.01032 | 0.0150 | 0.00425 | |
| 9 | 6.98 | 0.455 | 0.486 | 0.0417 | 0.032 | | | | | | | | | |
| 10 | 6.90 | 0.409 | 0.419 | 0.0525 | 0.043 | 37.1 | 0.210 | 0.349 | 0.078 | 23.2 | 0.00904 | 0.0150 | 0.00336 | |
| | | | | | | 34.4* | 0.217 | 0.421 | 0.086 | 22.8 | 0.00953 | 0.0185 | 0.00375 | |
| Total. | 53.08 | 3.277 | 3.346 | 0.3082 | 0.258 | 269.2 | 1.625 | 2.662 | 0.690 | 172.1 | | | | |
| Per day. | 5.90 | 0.364 | 0.372 | 0.0342 | 0.029 | 29.9 | 0.181 | 0.296 | 0.077 | 19.1 | 0.00944 | 0.0155 | 0.00401 | |
| 11 | 6.66 | 0.126 | 0.142 | 0.0109 | 0.007 | 28.4 | 0.158 | 0.305 | 0.083 | 17.0 | 0.00927 | 0.0180 | 0.00486 | Thyroparathyroidectomy, 10.20 a.m. April 11. |
| 12 | 7.06 | 0.116 | 0.127 | 0.0005 | 0.001 | | | | | | | | | |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 13 | 7.12 | 0.154 | 0.184 | 0.0004 | 0.001 | 54.2 | 0.379 | 0.560 | 0.148 | 34.1 | 0.01117 | 0.0165 | 0.00432 | Faint tremor. |
| 14 | 9.98 | 0.329 | 0.411 | 0.0005 | 0.001 | 68.2† | 0.429 | 0.683 | 0.184 | 44.0 | 0.00974 | 0.0155 | 0.00418 | " " |
| 15 | 9.03 | 0.475 | 0.408 | 0.0005 | 0.001 | | | | | | | | | " " |
| Total. | 39.85 | 1.200 | 1.272 | 0.0128 | 0.011 | 150.8 | 0.966 | 1.548 | 0.415 | 95.1 | | | | Dog killed 12.30 p.m. April 16. |
| Average.. | 7.97 | 0.240 | 0.254 | 0.0026 | 0.022 | 30.2 | 0.193 | 0.310 | 0.083 | 19.0 | 0.01016 | 0.0163 | 0.00436 | Serum contained 7.63 mg. calcium per 100 cc. |

* Because of the operation, the dog was fed at 1 p.m. instead of at 9 a.m. as ordinarily. These feces were voided between 1 p.m. and 3 p.m. Feces voided within 2 hours of feeding would, ordinarily, be included with the previous days output.

† Includes contents of large intestine, which, if the dog had not been killed, would have been collected as the next days feces.

night. For that reason, they have been included in the fore period.

The dog was killed at 12.30 p.m., April 16. He had been fed at 9 a.m. but had not defecated since then. Any feces voided after 11 a.m. would, ordinarily, have been included in the next days collection. For that reason, the total for the period has been divided by five, not by four, to obtain the daily average. That this procedure is correct is shown by the fact that the daily excretion of silica during the fore period is thus calculated to be 19.1 gm. and that after the operation is 19.0 gm. The agreement is within the limit of error of the determinations.

The average excretion of calcium after the operation was 0.310 gm., or 0.014 gm. greater than the average for the fore period. But this is not so great as the decrease in the excretion in the urine, so that the net effect is a lowering of the calcium excretion by an average of 0.0197 gm. per day. The fact that the calcium content of the feces was increased on the last day of the fore period makes it seem probable, that, if the parathyroids had not been removed, the calcium excretion in the feces would have continued to increase. This increase, presumably, would have been at the expense of the calcium in the urine, which would probably have fallen to the low level attained, a fact which was observed in other normal dogs on the same diet.

Be that as it may, the fact remains that the total calcium excretion was less after the operation than before.

*Magnesium.*—In the first two experiments, the excretion of magnesium was slightly increased. In the third experiment, the slight increase in the excretion in the feces was exactly balanced by a similar decrease in the excretion in the urine.

*Organic Phosphorus.*—In the tables, data are presented for inorganic phosphate and for total phosphorus. The differences are often considerable. As a rule, the total phosphorus is the greater, but this is not always the case. The differences, in normal urine, are probably due to errors in the determination of inorganic phosphate. The fact that the difference after operation is more uniformly in favor of the total phosphorus seems to indicate that some organic phosphorus may be present. Similar observations were made by one of us in 1911 (10).

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# THE EXCRETION OF CALCIUM, PHOSPHORUS, AND MAGNESIUM AFTER THE INJECTION OF CALCIUM CHLORIDE, SODIUM PHOSPHATE, OR BOTH.

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In the course of his work on the physiology of the parathyroid glands, Salvesen (1, 2) found that calcium, injected intravenously as the chloride, rapidly disappeared from the blood of parathyroidectomized dogs and, in two out of three experiments, was promptly eliminated, chiefly in the feces. Upon this observation and the earlier report by MacCallum and Voegtlin of an increased excretion of calcium after parathyroidectomy (3), Salvesen based his hypothesis of a lowered threshold for the excretion of calcium as the cause of the tetany of parathyroidectomized dogs.

In the preceding paper, the authors (4) have shown that there is no increased excretion of calcium after thyroparathyroidectomy but, rather, a decrease. This is, apparently, the effect of the lowered concentration in the blood. There is no evidence of the existence of a lowered threshold for the excretion of calcium, in the sense in which Salvesen employed the term. It is necessary, therefore, to find some other explanation for the rapid excretion of the injected calcium in Salvesen's experiments.

This is the more essential since Salvesen, Hastings, and McIntosh (5) found that the injection of calcium chloride into two normal dogs on a constant diet of minced meat was followed by a decrease in the amount of calcium excreted during the following 3 days. In one of the experiments, an after period of 3 additional days was also obtained. This showed a continued decrease in the amount excreted. This remarkable finding is not in accord with the previous results of Rüdel (6), Rey (7), Goitein (8), and Mendel and Benedict (9). It seems to us that the results of

Salvesen, Hastings, and McIntosh can be interpreted only as an indication of extreme calcium starvation, which the injected calcium was insufficient to satisfy.

The situation in Salvesen's parathyroidectomized dogs was quite different. These animals had received daily injections of calcium chloride for 11, 13, and 6 days, respectively, before the day upon which the rate of excretion of the injected calcium was determined. Salvesen seems to have assumed that all this previously injected calcium had been excreted but there is no evidence that this was the case. Unpublished observations of our own show that, after the administration of calcium to thyroparathyroidectomized dogs, there was a marked retention of calcium which might amount to several gm. in a few days. Obviously, the amount of retained calcium could not indefinitely increase at any considerable rate and, eventually, an equilibrium must have resulted.

It is, at least, suggestive that, out of the three dogs employed by Salvesen, the two showing the prompt, complete excretion of the injected calcium were the two that had previously received daily injections of calcium salts for 11 and 13 days, respectively, and that the one that had previously received such injections for only 6 days did not show this complete, rapid excretion of the calcium injected on the 7th day.

We are now of the opinion that this overloading of the body with calcium was responsible for the prompt excretion of the injected calcium in Salvesen's experiments. However, there is another factor that may be of significance and in which we were, before our observations on calcium retention in thyroparathyroidectomized dogs, inclined to find the explanation for Salvesen's results.

After parathyroidectomy, there is a very marked retention of phosphate (4, 10–12) (see also foot of Table V of this paper). Salvesen did not determine the excretion of phosphate in his experiments but, from our experience, it is quite apparent that the amount retained was probably more than sufficient to combine with all the injected calcium to form $Ca_3(PO_4)_2$.

It is quite impossible, from the published protocols, to determine the amount of calcium received by Salvesen's Dog 10[1]

[1] Salvesen (2), pp. 43 and 51.

but, if we assume it to have been the same as on the day on which the rate of excretion of the injected calcium was determined, or 2 gm. $CaCl_2$, we find this to have been the equivalent of 0.373 gm. phosphorus or 0.0143 gm. per kilo. Salvesen's Dog 11[2] received not more than 2 gm. $CaCl_2$ per day. This is the equivalent of 0.0257 gm. phosphorus per kilo. And his Dog 12[3] received, in the 7 days that closed with the days on which the excretion of calcium was determined, 4.8 gm. of calcium chloride and 3 gm. of calcium lactate, the equivalent of a total of 2.13 gm. of calcium, which is the equivalent of 0.0102 gm. of phosphorus per kilo per day. We have repeatedly obtained even greater retentions of phosphorus. Therefore, it seemed to us not unlikely that the excretion of the injected calcium after parathyroidectomy might be accelerated by the presence of phosphate.

If this hypothesis is correct, the administration of phosphate, together with the calcium chloride, should accelerate the excretion of the injected calcium. This was found to be the case. When calcium chloride was injected alone, most of the calcium was retained. When injected together with sodium phosphate, the increase in the excretion of calcium was equal to the amount injected. Unfortunately, the significance of the results is not absolutely clear because the simultaneous injection of the two solutions was followed by a marked increase in the excretion of nitrogen. The amount of "excess" phosphorus excreted was greater than the amount injected. Obviously, some was derived from the increased endogenous metabolism indicated by the increased nitrogen excretion. It is difficult to evaluate the amount of calcium that was liberated as a result of this metabolism and, consequently, impossible to determine just how much of the "excess" calcium excreted was derived from that injected. However, it is scarcely conceivable that the increased metabolism should have been responsible for the excretion of sufficient calcium to materially affect the conclusions.

In another experiment, the effect of the injection of sodium phosphate, alone, was determined. Although the amount of phosphate injected was greater, the increase in the excretion of nitrogen was less than when calcium chloride was also adminis-

[2] Salvesen (2), pp. 44 and 53.
[3] Salvesen (2), pp. 45, 55, and 62.

TABLE I.

*Effect of the Injection of Calcium Chloride upon the Excretion of Nitrogen, Phosphorus, Calcium, and Magnesium.*

Dog 30, weight 19.5 kilos. Fed, daily, a mixture of 52 gm. dried beef heart, 69 gm. cracker meal, 69 cc. maize oil, 26 gm. infusorial earth, and 1000 cc. water.

| Date. | Urine. | | | | | Feces. | | | | | | | | Remarks. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Nitrogen. | Phosphorus. | | Calcium. | Magnesium. | Total. | Phosphorus. | Calcium. | Magnesium. | Silica. | Per gm. silica. | | | |
| | | Inorganic. | Total. | | | | | | | | Phosphorus. | Calcium. | Magnesium. | |
| *1924* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *mg.* | *mg.* | *mg.* | |
| Dec. | | | | | | | | | | | | | | |
| 20 | 5.54 | 0.422 | 0.413 | 0.0003 | 0.037 | 25.8 | 0.114 | 0.181 | 0.051 | 18.6 | 6.13 | 9.74 | 2.72 | |
| 21 | 6.37 | 0.458 | 0.468 | 0.0002 | 0.062 | 33.5 | 0.169 | 0.292 | 0.079 | 22.2 | 7.63 | 13.16 | 3.54 | |
| 22 | 5.72 | 0.405 | 0.380 | 0.0002 | 0.048 | 31.0 | 0.129 | 0.210 | 0.059 | 20.7 | 6.38 | 10.11 | 2.86 | |
| 23 | 3.01 | 0.146 | 0.152 | 0.0001 | 0.012 | | | | | | | | | |
| Total. | 20.64 | 1.431 | 1.413 | 0.0008 | 0.159 | 90.3 | 0.412 | 0.683 | 0.189 | 61.5 | | | | Feces total and average for 3 days, only. |
| Average. | 5.16 | 0.358 | 0.353 | 0.0002 | 0.040 | 30.1 | 0.138 | 0.228 | 0.063 | 20.5 | 6.70 | 11.11 | 3.07 | |
| 24,A | 2.59 | 0.237 | 0.257 | 0.0520 | 0.050 | | | | | | | | | Injected 31 cc. $CaCl_2$ = 0.76 gm. Ca and 100 cc. 0.8 per cent NaCl, 9.30 to 10 a.m. A voided at 1 p.m., B during rest of day. |
| 24,B | 3.76 | 0.213 | 0.205 | 0.0047 | 0.034 | 50.7 | 0.333 | 0.553 | 0.120 | 34.9 | 9.53 | 15.82 | 3.42 | |
| 25 | 7.20 | 0.496 | 0.488 | 0.0002 | 0.057 | 19.9 | 0.112 | 0.205 | 0.043 | 13.2 | 8.49 | 15.44 | 3.25 | |
| 26 | 5.38 | 0.429 | 0.423 | 0.0003 | 0.032 | 37.0 | 0.160 | 0.297 | 0.067 | 25.5 | 6.28 | 11.65 | 2.63 | |
| 27 | 4.69 | 0.293 | 0.288 | 0.0002 | 0.028 | 31.6 | 0.157 | 0.293 | 0.062 | 21.5 | 7.33 | 13.65 | 2.87 | |
| 28 | 4.92 | 0.299 | 0.299 | 0.0001 | 0.029 | 53.2 | 0.271 | 0.486 | 0.089 | 38.8 | 7.00 | 12.54 | 2.29 | |
| 29 | 5.87 | 0.324 | 0.316 | 0.0002 | 0.036 | 18.7 | 0.070 | 0.147 | 0.026 | 13.8 | 5.08 | 10.62 | 1.90 | |
| 30 | 4.66 | 0.323 | 0.338 | 0.0002 | 0.040 | 31.0 | 0.117 | 0.220 | 0.047 | 22.6 | 5.19 | 9.73 | 2.10 | |
| Total. | 39.07 | 2.614 | 2.614 | 0.0579 | 0.306 | 242.1 | 1.220 | 2.201 | 0.454 | 170.3 | | | | Feces include 1 day of fore period. |
| Average. | 5.58 | 0.373 | 0.373 | 0.0083 | 0.044 | 30.3* | 0.153* | 0.275* | 0.057* | 21.3* | 7.16 | 12.92 | 2.67 | |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 31 Jan. | 4.88 | 0.333 | 0.316 | 0.0004 | 0.036 | 30.0 | 0.128 | 0.225 | 0.054 | 21.2 | 6.01 | 10.59 | 2.53 |
| 1 | 4.72 | 0.325 | 0.326 | 0.0002 | 0.047 | 46.3 | 0 195 | 0 416 | 0 082 | 32.9 | 5.94 | 12.64 | 2.49 |
| 2 | 6.55 | 0.470 | 0.438 | 0.0003 | 0.035 | 24.2 | 0.099 | 0 148 | 0.043 | 16.7 | 5.90 | 8.87 | 2.57 |
| Total. | 16.15 | 1.128 | 1.080 | 0.0009 | 0.118 | 100.5 | 0.422 | 0.789 | 0.179 | 70.8 | | | |
| Average. | 5.38 | 0.376 | 0.360 | 0.0003 | 0.039 | 33.5 | 0.141 | 0.263 | 0.060 | 23.6 | 5.96 | 11.15 | 2.53 |

* The dog passed no feces on Dec. 23, so that those properly belonging to this day were carried over into the experimental period. The dog was not catheterized before the injection and some of the urine obtained on the 24th was secreted during the fore period. After the injection, the dog vomited saliva and mucus. Some of this contaminated the urine but most of it was collected, added to the food, and taken eagerly at 10.45 a.m.

tered. There was a marked increase in the excretion of calcium. This continued for several days, apparently reaching a maximum about 7 days after the injection. Unfortunately, the feces were not analyzed promptly, the continuance of the increased calcium excretion was not realized, and the animal was used for another experiment.

A similar increase in the excretion of calcium in the feces, without appreciable change in the output in the urine, was observed by Jeppsson (13) in children after the oral administration of $Na_2HPO_4$ or of $K_2HPO_4$.

It would seem, from the results we have obtained, that the rate of excretion of calcium and, probably, of phosphate, after administration of either depends upon the amount of the other administered at the same time, with the food or otherwise, and, probably, upon the previous store of both in the body. These factors, as well as the possible increase in endogenous metabolism after injection, seem to have been neglected by most previous investigators.

In order to detect possible increases in the amount of organic phosphorus in the urine, we regularly determined the total phosphorus by oxidation with sulfuric and nitric acids and precipitation with molybdate solution and also determined the inorganic phosphate by Brigg's method (14). As is evident from the tables, there were irregular differences between the two. Sometimes the values for inorganic phosphate were higher than those for total phosphorus. We believe that the differences were due to the presence of substances of unknown nature that reacted with the reagents to increase, or to decrease, the color. There was no satisfactory evidence for the presence of an appreciable quantity of an organic phosphorus compound.

## EXPERIMENTAL.

The manner of feeding the dogs and the methods of analyzing the excreta were the same as in the experiments in the previous paper (4).

The first experiment of the present series (Table I) was intended to determine the effect of the injection of calcium chloride alone. The excretion of nitrogen and phosphorus in the urine during the experimental period showed a slight increase, which was, however, apparent and not real, because some of the urine of the fore

period was carried over into the experimental period. The ratio of phosphorus to nitrogen was practically unchanged. The excretion of magnesium in the urine was slightly increased, particularly immediately after the injection. The excretion of calcium in the urine was negligible, except on the day of the injection.

The average daily excretion of calcium in the feces was increased after the injection, and, at first sight, seems not to have decreased in the after period. But examination of the figures for the amount of calcium excreted per gm. of silica in the feces shows that these were practically identical in the fore and after periods, 11.11 and 11.15 mg. per gm., respectively. The explanation is found in

TABLE II.

*Comparison of the Amounts of Phosphorus and Calcium Actually Excreted in the Feces after the Injection of Calcium Chloride with those Calculated from the Excretion during the Fore Period.*

All data cumulative. See also Table I and text, page 208.

| Date | Phosphorus. | | | Calcium. | | |
|---|---|---|---|---|---|---|
| | Excreted. | Calculated. | Excess. | Excreted. | Calculated. | Excess. |
| *1924* Dec. | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* |
| 24 | 0.166 | 0.145 | 0.021 | 0.276 | 0.240 | 0.036 |
| 25 | 0.278 | 0.290 | 0.012 | 0.481 | 0.480 | 0.001 |
| 26 | 0.438 | 0.435 | 0.003 | 0.778 | 0.720 | 0.058 |
| 27 | 0.595 | 0.580 | 0.015 | 1.071 | 0.960 | 0.109 |
| 28 | 0.866 | 0.725 | 0.141 | 1.557 | 1.200 | 0.357 |
| 29 | 0.936 | 0.870 | 0.066 | 1.704 | 1.440 | 0.264 |
| 30 | 1.089 | 1.015 | 0.074 | 1.990 | 1.680 | 0.310 |

the overlapping of the periods. The total amount of silica excreted in the 14 days of the entire experiment was 302.6 gm., an average of 21.61 gm. per day. The average for the 3 day fore period was 20.5 gm., that for the after period was 23.6 gm. Evidently, the former figure is too low and the latter too large. The value given in the table for the total excretion of calcium during the fore period is, therefore, too low by the amount carried over into the experimental period. The correct amount may be obtained by multiplying the average daily excretion of silica (for the entire experiment) by the amount of calcium per gm. of silica in feces of the period. The corrected value for the average daily

excretion of calcium in both fore and after periods becomes 0.241 gm.

A precisely similar calculation applied to the figures for the phosphorus excretion makes the corrected amount for the 3 days of the fore period 0.145 gm. per day and, for the after period, 0.129 gm. per day. These are not identical but examination of the figures for the amount of phosphorus per gm. of silica in the feces shows that this was increased considerably after the injection, to fall thereafter, reaching its lowest value at the end of the experimental period, then rising, but not reaching its original value even 8 days after the injection. The corrected amount of phosphorus excreted in the feces of the experimental period was 1.089 gm. That calculated from the rate per day during the fore period was 1.015, indicating an increased excretion, due to the injection of 0.070 gm. This is somewhat more than the amount (0.048 gm.) required to bring the amount excreted during the after period up to that calculated from the rate during the fore period. In other words, the increased excretion of phosphorus during the first 5 days after the injection had almost, but not quite, been compensated for at the end of the experiment.

The amount of magnesium excreted per gm. of silica was 3.07 mg. in the fore period, 2.67 mg. in the experimental period, and 2.53 mg. in the after period. Apparently, there was some continuous process at work, tending to lower the amount of magnesium excreted in the feces.

For the purpose of calculating the increases due to the injection, we have compared the amounts of phosphorus and calcium actually excreted in the feces, after correction for the overlapping at the beginning and end of the experimental period, with those calculated by multiplying the number of days by the figures for the normal excretion, that during the fore period. The figures obtained are presented in Table II and show that not more than 0.31 or 0.35 gm. of the 0.760 gm. of calcium injected was excreted in the feces within the 6 days following the injection. With the addition of the amount of calcium excreted in the urine, the maximum total excretion was 0.40 gm. Since the rate of excretion in both urine and feces in the after period was the same as in the fore period, the rest of the calcium must have been deposited, in fairly stable form, in the tissues.

The figures in Table II also show that the excess calcium in the feces was not accompanied by enough excess phosphorus to form $Ca_3(PO_4)_2$. Some of the excess calcium was probably excreted in combination with fatty acid or with carbonic acid.

In the next experiment, a similar quantity of calcium chloride was injected but this was immediately preceded by an equivalent quantity of neutral sodium phosphate. There was a marked increase in the amount of nitrogen in the urine. The same result was observed in a similar, unpublished, experiment. The phosphorus content of the urine was also increased but the ratio of phosphorus to nitrogen was lower in the experimental period than in either the fore or after periods. The excretion of calcium in the urine was negligible up to the time of the injection, when it increased very considerably. It then gradually diminished, again becoming negligible on the 8th day. The excretion of magnesium in the urine was increased after the injection, returning to the normal in the after period.

In the feces, there was a very evident increase in the excretion of phosphorus, calcium, and magnesium. Calculation of the exact amount is complicated by the overlapping of the periods. The total amount of silica excreted in the 20 days of this and the following experiment was 375.5 gm., an average of 18.78 gm. Since the average amount collected in the fore period was only 15.6 gm. per day, while that in the experimental period was 21.7 gm., it is quite evident that some of the feces properly belonging in the fore period were carried over into the experimental period. The low value for the silica of the after period (March 8 to 10) seems to have been due to an unusually prompt evacuation on March 7. Just as in the preceding experiment, the corrected average daily excretion of phosphorus, calcium, and magnesium in the feces may be calculated by multiplying the average daily output of silica during the entire experiment by the amounts of these substances per gm. of silica in the feces of the respective periods. The corrected values are 0.171 gm. phosphorus, 0.302 gm. calcium, and 0.078 gm. magnesium per day during the fore period and 0.158 gm. phosphorus, 0.284 gm. calcium, and 0.060 gm. magnesium during the after period. The daily excretion of each of these substances was less in the after period than in the fore period. Therefore, in calculating the figures in Table IV,

TABLE III.

*Effect of the Injection of Calcium Chloride and Sodium Phosphate, Together upon the Excretion of Nitrogen, Phosphorus, Calcium, and Magnesium.*

Dog 32, weight 17.5 kilos. Fed daily a mixture of 46 gm. dried beef heart, 62 gm. cracker meal, 62 cc. maize oil, 24 gm. infusorial earth, and 1000 cc. water.

| Date. | Urine. | | | | | Feces. | | | | | | | | Remarks. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Nitrogen. | Phosphorus. | | Calcium. | Magnesium. | Total. | Phosphorus. | Calcium. | Magnesium. | Silica. | Per gm. silica. | | | |
| | | Inorganic. | Total. | | | | | | | | Phosphorus. | Calcium. | Magnesium. | |
| *1925* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *mg.* | *mg.* | *mg.* | |
| Feb. | | | | | | | | | | | | | | |
| 27 | 6.16 | 0.420 | 0.424 | 0.0001 | 0.050 | 28.7 | 0.156 | 0.265 | 0.060 | 16.4 | 9.56 | 16.2 | 3.7 | |
| 28 | 6.39 | 0.370 | 0.403 | 0.0002 | 0.045 | 16.5 | 0.101 | 0.165 | 0.038 | 10.1 | 10.02 | 16.3 | 3.7 | |
| Mar. | | | | | | | | | | | | | | |
| 1 | 2.78 | 0.069 | 0.074 | 0.0001 | 0.005 | 33.9 | 0.170 | 0.323 | 0.078 | 20.3 | 8.34 | 15.9 | 3.8 | |
| 1,B | 2.53 | 0.273 | 0.267 | 0.0141 | 0.033 | | | | | | | | | Voided immediately after injection, Mar. 2. |
| Total. | 17.86 | 1.132 | 1.168 | | 0.133 | 79.1 | 0.427 | 0.753 | 0.176 | 46.8 | | | | |
| Average. | 5.95 | 0.377 | 0.389 | 0.0001* | 0.044 | 26.4 | 0.142 | 0.251 | 0.059 | 15.6 | 9.12 | 16.1 | 3.8 | |
| 2 | 6.69 | 0.438 | 0.479 | 0.0942 | 0.046 | 40.8 | 0.249 | 0.508 | 0.107 | 20.9 | 11.93 | 24.3 | 5.1 | Injected 32 cc. neutral sodium phosphate (containing 0.356 gm. P), 60 cc. 0.8 per cent NaCl, 75.5 cc. $CaCl_2$ (containing 0.758 gm. Ca), 60 cc. 0.8 per cent. |
| 3 | 9.42 | 0.498 | 0.489 | 0.0159 | 0.007 | 44.1 | 0.214 | 0.398 | 0.080 | 19.2 | 11.13 | 20.7 | 4.2 | |
| 4 | 12.38 | 0.711 | 0.806 | 0.0336 | 0.101 | 50.1 | 0.356 | 0.562 | 0.154 | 28.3 | 12.58 | 19.9 | 5.4 | |
| 5 | 4.33 | 0.237 | 0.219 | 0.0255 | 0.029 | 31.6 | 0.183 | 0.316 | 0.064 | 18.7 | 9.76 | 16.9 | 3.4 | |
| 6 | 7.27 | 0.322 | 0.332 | 0.0122 | 0.058 | 31.1 | 0.159 | 0.317 | 0.059 | 18.7 | 8.52 | 17.0 | 3.2 | |
| 7 | 9.28 | 0.492 | 0.497 | 0.0161 | 0.074 | 38.6 | 0.207 | 0.460 | 0.090 | 24.2 | 8.55 | 19.0 | 3 7 | |
| Total. | 49.37 | 2.698 | 2.822 | 0.2290† | 0.315 | 236.3 | 1.368 | 2.561 | 0.554 | 130.0 | | | | |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Average. | 8.23 | 0.450 | 0.470 | 0.0286† | 0.053 | 39.4 | 0.228 | 0.427 | 0.092 | 21.7 | 10.52 | 19.7 | 4.3 | NaCl between 10.00 and 10.25 a.m. Urine contains some vomit. |
| 8 | | | | | | | | | | | | | | |
| 9 | 10.66 | 0.562 | 0.563 | 0.0174 | 0.075 | 48.1 | 0.231 | 0.411 | 0.108 | 31.0 | 7.46 | 13.3 | 3.5 | |
| 10 | 6.50 | 0.541 | 0.492 | 0.0003 | 0.060 | 40.7 | 0.226 | 0.412 | 0.065 | 23.4 | 9.67 | 17.7 | 2.7 | |
| Total. | 17.16 | 1.103 | 1.055 | | 0.135 | 88.8 | 0.457 | 0.823 | 0.173 | 54.4 | | | | |
| Average. | 5.72 | 0.368 | 0.352 | | 0.045 | 29.6 | 0.152 | 0.274 | 0.058 | 18.1 | 8.40 | 15.1 | 3.2 | |

* Does not include urine Mar. 1,B.

† Includes Mar. 1,B, and 8–9.

which shows the rate of elimination of "excess" phosphorus, calcium, and magnesium in the feces, the values for the normal excretion have been calculated from the average of those observed during the fore and after periods.

It is obvious from the table that the excretion of calcium in the *feces* after the injection of calcium chloride and sodium phosphate was far greater than was observed after the injection of calcium chloride alone. In fact, the *total* additional amount excreted in the urine and feces was exactly equal to the amount injected. The increase in the phosphorus excretion was considerably greater than the amount injected.

TABLE IV.

*Comparison of the Amounts of Phosphorus, Calcium, and Magnesium Actually Excreted in the Feces after the Injection of Calcium Chloride and Sodium Phosphate with Those Calculated from the Excretion during Fore Period.*

All data cumulative. See also Table III and text, page 209.

| Date. | Phosphorus. | | | Calcium. | | | Magnesium. | | |
|---|---|---|---|---|---|---|---|---|---|
| | Excreted. | Calculated. | Excess. | Excreted. | Calculated. | Excess. | Excreted. | Calculated. | Excess. |
| *1925* Mar. | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* |
| 2 | 0.162 | 0.165 | −0.003 | 0.354 | 0.293 | 0.061 | 0.071 | 0.069 | 0.002 |
| 3 | 0.376 | 0.330 | 0.046 | 0.752 | 0.586 | 0.166 | 0.151 | 0.138 | 0.013 |
| 4 | 0.732 | 0.495 | 0.237 | 1.314 | 0.879 | 0.435 | 0.305 | 0.207 | 0.098 |
| 5 | 0.915 | 0.660 | 0.255 | 1.630 | 1.172 | 0.458 | 0.369 | 0.267 | 0.093 |
| 6 | 1.074 | 0.825 | 0.259 | 1.947 | 1.465 | 0.482 | 0.428 | 0.345 | 0.083 |
| 7 | 1.215 | 0.990 | 0.225 | 2.289 | 1.758 | 0.531 | 0.493 | 0.414 | 0.079 |

It may be objected that calcium might have been derived from the tissue destruction indicated by the increased excretion of nitrogen during the experimental period. Most of this nitrogen was, in all probability, derived from muscular tissue. The total "excess" nitrogen in the urine was 13.67 gm. According to Sherman (15) average meat contains, per 100 gm. protein, 0.058 gm. calcium, and 1.078 gm. phosphorus. Assuming these ratios to hold for dog muscle, this amount of nitrogen is the equivalent of 0.907 gm. phosphorus and of 0.048 gm. calcium. The amount of phosphorus excreted in excess of the sum of the normal excretion and the amount injected was 0.468 gm. According to Sherman's figures, this, if derived from muscle, would be the equivalent of

TABLE V.

*Effect of the Injection of Sodium Phosphate on the Excretion of Nitrogen, Phosphorus, Calcium, and Magnesium.*

Dog 32, continued from Table III.

| Date. | Urine. | | | | | Feces. | | | | | | | | Remarks. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Nitrogen. | Phosphorus. | | Calcium. | Magnesium. | Total. | Phosphorus. | Calcium. | Magnesium. | Silica. | Per gm. silica. | | | |
| | | Inorganic. | Total. | | | | | | | | Phosphorus. | Calcium. | Magnesium. | |
| *1925* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *mg.* | *mg.* | *mg.* | |
| Mar. 8–10 | 5.72 | 0.368 | 0.352 | 0.0003 | 0.045 | 29.6 | 0.152 | 0.274 | 0.058 | 18.1 | 8.40 | 15.13 | 3.2 | Corrected values, see page 209. |
| Average. | | | | | | | 0.158 | 0.284 | 0.060 | 18.78 | | | | |
| 11 | 5.49 | 0.775 | 0.754 | 0.0013 | 0.013 | | | | | | | | | Injected 75 cc. sodium phosphate, pH 7.4, containing 0.834 gm. P. Dog vomited contaminating urine and feces. Analyses on mixtures. Dog fed after vomiting ceased. |
| 12 | 8.44 | 0.644 | 0.587 | 0.0038 | 0.066 | 64.1 | 0.373 | 0.766 | 0.131 | 39.0 | 9.56 | 19.66 | 3.4 | |
| 13 | 7.69 | 0.462 | 0.545 | 0.0159 | 0.062 | 23.5 | 0.147 | 0.241 | 0.074 | 13.6 | 10.80 | 17.71 | 5.5 | |
| 14 | 6.78 | 0.428 | 0.464 | 0.0026 | 0.052 | 44.8 | 0.262 | 0.482 | 0.098 | 26.2 | 10.00 | 18.41 | 3.7 | |
| 15 | 4.13 | 0.209 | 0.244 | 0.0004 | 0.021 | | | | | | | | | |
| 16 | 5.28 | 0.295 | 0.310 | 0.0002 | 0.042 | 47.7 | 0.269 | 0.536 | 0.121 | 27.8 | 9.67 | 19.25 | 4.3 | |
| 17 | 6.02 | 0.413 | 0.470 | 0.0004 | 0.055 | 38.1 | 0.230 | 0.482 | 0.076 | 23.1 | 10.04 | 20.90 | 3.3 | |
| 18 | 7.76 | 0.597 | 0.665 | 0.0161 | 0.072 | 27.9 | 0.177 | 0.324 | 0.079 | 14.6 | 12.28 | 22.18 | 5.4 | |
| Total. 11–18. | 51.59 | 3.823 | 4.039 | 0.0405 | 0.383 | 246.1 | 1.458 | 2.831 | 0.579 | 144.3 | | | | |
| Average. | 6.45 | 0.478 | 0.505 | 0.0051 | 0.048 | 30.8 | 0.182 | 0.354 | 0.073 | 18.0 | 10.11 | 19.61 | 4.0 | |
| 19 | 5.99 | | 0.008 | 0.0124 | 0.022 | 14.3 | 0.90 | 0.160 | 0.047 | 8.2 | 10.94 | 19.47 | 5.7 | Thyroparathyroidectomy. |

only 0.025 gm. calcium. Aron and Gralka (16) state that the values in the literature for the calcium content of dog muscle vary from 0.050 to 0.189 gm. per 1000 gm. of fresh muscle. Assuming the latter to contain about 2.5 per cent nitrogen, the loss of 13.67 gm. of nitrogen might involve the loss of from 0.027 to 0.100 gm. of calcium.

It would be necessary to assume that almost one-half of the increased metabolic phosphorus was derived from bone, or other tissue with the same calcium: phosphorus ratio, to reduce the increase in calcium excretion ascribable to the injection to the amount found in the previous experiment. Consequently, there seems to be little doubt but that the rate of excretion of the injected calcium was accelerated by the simultaneous injection of phosphate.

The experiment just described was immediately followed by another in which sodium phosphate, alone, was injected. The amount was much greater than the amount used in conjunction with calcium chloride. This produced vomiting and diarrhea. The analyses for that day are based upon urine and feces that were contaminated with vomit. The latter did not contain any food particles for the dog was not fed until 2 hours after vomiting had ceased. Thereafter, the dog behaved normally and feces of usual consistency were obtained. No other symptoms, certainly none resembling tetany, were observed.

The effect of the injection upon the excretion of nitrogen, phosphorus, calcium, and magnesium is shown in Table V. The injection of the phosphate solution was followed by an increase in the phosphate excretion in both urine and feces. The total increase was greater than the amount injected by at least 0.50 gm. but the excretion of nitrogen in the urine was also increased by about 5.8 gm. The average excretion of silica is a little low, apparently, because of failure to secure complete collection on the last day. The excretion of calcium in both urine and feces was increased as was also the excretion of magnesium in the feces. Unfortunately, the experiment was not continued for a sufficiently long time.

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# THE EFFECT OF THE ADMINISTRATION OF A POTENT PARATHYROID EXTRACT UPON THE EXCRETION OF NITROGEN, PHOSPHORUS, CALCIUM, AND MAGNESIUM, WITH SOME REMARKS ON THE SOLUBILITY OF CALCIUM PHOSPHATE IN SERUM AND ON THE PATHOGENESIS OF TETANY.

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In a previous communication (1), the authors have shown that there is not, after parathyroidectomy in dogs, an increased excretion of calcium. It became necessary, therefore, to find some other explanation for the lowered concentration of calcium in the serum of such dogs. Experiments which were designed to furnish such explanation were planned and were in progress[1] when Collip (2) reported the preparation, from parathyroid glands, of an extract the administration of which relieved the tetany of parathyroidectomized dogs and increased the concentration of calcium in the serum in both normal and parathyroidectomized dogs.

The use of such an extract offered a new method for investigating the relation between the parathyroid glands and the metabolism of calcium and phosphorus. Many problems suggested themselves but it seemed essential to first determine what effect, if any, the administration of such an extract and the high concentration of calcium in the plasma that it caused, had upon the excretion of calcium and phosphorus. Dr. Collip very kindly sent us a generous supply of an extract containing 10 units per cc., the unit being 0.01 of the amount that would produce an

[1] These experiments have been completed and will shortly be reported.

TABLE I.

*Effect of the Administration of Parathyroid Extract upon the Excretion of Nitrogen, Phosphorus, Calcium, and Magnesium.*

Dog 35, young female, weight 5.4 kilos. Fed daily a mixture of 14 gm. dried beef heart, 18 gm. cracker meal, 18 cc. maize oil, 8 gm. infusorial earth, and 250 cc. water.

| Date. | Urine. | | | | Feces. | | | | | | | | Remarks. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | Per gm. silica. | | | |
| | Nitrogen. | Phosphorus. | Calcium. | Magnesium. | Weight. | Phosphorus. | Calcium. | Magnesium. | Silica. | Phosphorus. | Calcium. | Magnesium. | |
| *1918* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *mg.* | *mg.* | *mg.* | |
| May | | | | | | | | | | | | | |
| 11–13 | 5.45 | 0.464 | 0.0008 | 0.035 | 24.6 | 0.0816 | 0.0954 | 0.060 | 13.8 | 5.92 | 6.92 | 4.45 | |
| 14–15 | 3.40 | 0.262 | 0.0005 | 0.022 | 24.0 | 0.0730 | 0.0819 | 0.068 | 14.2 | 5.14 | 5.76 | 4.79 | |
| 16–17 | 3.73 | 0.300 | 0.0008 | 0.022 | 18.2 | 0.0500 | 0.0588 | 0.070 | 10.5 | 4.76 | 5.60 | 6.65 | |
| Total. | 12.58 | 1.026 | 0.0021 | 0.079 | 66.8 | 0.2046 | 0.2361 | 0.198 | 38.5 | | | | |
| Average. | 1.80 | 0.147 | 0.0003 | 0.011 | 9.54 | 0.0292 | 0.0337 | 0.028 | 5.5 | 5.31 | 6.13 | 5.14 | |
| 18–19 | 4.08 | 0.472 | 0.0618 | 0.049 | 18.9 | 0.0480 | 0.0535 | 0.062 | 10.5 | 4.58 | 5.11 | 5.96 | 1 cc. parathyroid extract twice each day. |
| 20–21 | 3.18 | 0.270 | 0.0590 | 0.022 | 13.2 | 0.0522 | 0.0660 | 0.062 | 7.3 | 7.20 | 9.11 | 8.60 | 1 cc. parathyroid extract 9.10 a.m.; 5.20 and 11.20 p.m., May 20. |
| 21 (vomit) | 1.10 | | | | | 0.0748 | 0.0001 | 0.002 | 1.1 | | | | 1 cc. parathyroid extract 9 a.m., May 21, fed 9 a.m. Vomited, |
| 22 | 2.82 | 0.282 | 0.0034 | 0.011 | 11.8 | 0.0524 | 0.0794 | 0.043 | 6.5 | 8.04 | 12.2 | 6.60 | 12.30, contaminating urine. |
| 23–24 | 5.71 | 0.499 | 0.0063 | 0.024 | 16.7 | 0.0800 | 0.1200 | 0.084 | 9.4 | 8.49 | 12.7 | 8.92 | Sample of blood taken at 2.30 |
| 25–26 | 4.86 | 0.383 | 0.0003 | 0.024 | 18.4 | 0.0935 | 0.1662 | 0.089 | 10.2 | 9.17 | 16.3 | 8.73 | p.m. Serum contained 16.6 |
| 27–28 | 4.13 | 0.336 | 0.0003 | 0.024 | 20.6 | 0.1160 | 0.1747 | 0.109 | 11.4 | 10.17 | 15.3 | 9.58 | mg. Ca per 100 cc. |
| 29–30 | 3.99 | 0.270 | 0.0004 | 0.010 | 18.4 | 0.1018 | 0.1664 | 0.061 | 10.4 | 9.79 | 16.0 | 5.84 | |
| 31–1 | 3.67 | 0.278 | 0.0004 | 0.020 | 19.2 | 0.0646 | 0.1163 | 0.063 | 11.1 | 5.84 | 10.5 | 5.73 | |
| Total. | 33.54 | 2.790 | 0.1319 | 0.184 | 147.2 | 0.6833 | 0.9426 | 0.575 | 77.9 | | | | |
| Average. | 2.25 | 0.186 | 0.0088 | 0.012 | 9.81 | 0.0456 | 0.0628 | 0.038 | 5.19 | 8.78 | 12.12 | 7.39 | |
| June 2 | 2.08 | 0.009 | 0.0004 | 0.008 | 9.3 | 0.0351 | 0.0488 | 0.028 | 5.13 | 6.84 | 9.50 | 5.53 | Thyroparathyroidectomy 11 a.m. |

average increase of 5 mg. in the calcium content of the blood serum in a normal dog weighing 20 kilos over a period of 15 hours. This extract was used in all our experiments.

These were conducted in the manner usual in this laboratory and the methods of analysis were those described in a previous communication (1). In the first experiment of the present series (Table I), urine and feces were collected in periods of 2 or 3 days each. The amount of calcium and phosphorus in the feces decreased steadily during the fore periods. This was not realized at the time and, before the excretion of these elements had reached a constant level, the administration of parathyroid extract was begun. 1 cc. was injected, subcutaneously, twice a day for 2 days and three times on the 3rd day. On the following day, only one injection was given because symptoms of overdosage appeared. The dog vomited, contaminating some of the urine. This mixture of urine and vomit was dried and analyzed. The dog then received her usual diet for 11 days after which the thyroids and parathyroids were removed, with the usual marked fall in the phosphorus excretion.

The excretion of calcium in the urine, which had been negligible before the administration of parathyroid extract, increased promptly, to decrease just as promptly as soon as the injections were stopped.

The amount of nitrogen in the urine increased from an average of 1.80 gm. per day to an average of 2.25 gm. per day, the maximum 3.18 gm. being reached on the 3rd day of the injections, with a gradual decrease after these were discontinued. The phosphorus excretion was also increased. Although the ratio of phosphorus to nitrogen was nearly the same in the experimental period as in the fore period, the ratio during the days of parathyroid administration was increased. Apparently, the excretion of calcium was accompanied by an increased excretion of phosphorus that was independent of the increase due to increased protein metabolism.

The amounts of phosphorus and of calcium in the feces of the first period after the administration of parathyroid extract was begun were less than in the fore period. Apparently, most of the feces passed in these 2 days were actually formed during the fore period and represent the excretion during that time. In

| June 2 | 2.08 | 0.009 | 0.0004 | 0.008 | 9.3 | 0.0351 | 0.0488 | 0.028 | 5.13 | 6.84 | 9.50 | 5.53 | Thyroparathyroidectomy 11 a.m. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

TABLE II.

*Effect of Administration of Parathyroid Extract upon the Excretion of Nitrogen, Phosphorus, Calcium, and Magnesium.*

Dog 34, male, weight 11.3 kilos. Fed daily a mixture of 30 gm. dried beef heart, 30 gm. cracker meal, 30 cc. maize oil, 15 gm. infusorial earth, and 500 cc. $H_2O$. Additional water *ad libitum*.

| Date. | Urine. | | | | Feces. | | | | | | | | Remarks. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Nitrogen. | Phosphorus. | Calcium. | Magnesium. | Weight. | Phosphorus. | Calcium. | Magnesium. | Silica. | Per gm. silica. Phosphorus. | Per gm. silica. Calcium. | Per gm. silica. Magnesium. | |
| *1925* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *mg.* | *mg.* | *mg.* | |
| June | | | | | | | | | | | | | |
| 18 | 4.58 | 0.199 | 0.0002 | 0.023 | 33.6 | 0.264 | 0.323 | 0.150 | 21.8 | 12.1 | 14.8 | 6.9 | |
| 19 | 3.93 | 0.186 | 0.0002 | 0.019 | | | | | | | | | |
| 20 | | | | | 15.3 | 0.124 | 0.127 | 0.043 | 9.3 | 13.3 | 13.6 | 4.6 | |
| 21 | 7.59 | 0.393 | 0.0003 | 0.046 | 12.7 | 0.106 | 0.161 | 0.038 | 8.2 | 13.0 | 19.7 | 4.7 | |
| 22 | 2.07 | 0.037 | 0.0003 | 0.004 | 33.7 | 0.304 | 0.381 | 0.061 | 20.6 | 14.7 | 18.5 | 2.9 | |
| 23 | 4.79 | 0.307 | 0.0003 | 0.031 | | | | | | | | | |
| 24 | 5.66 | 0.239 | 0.0005 | 0.036 | 20.0 | 0.153 | 0.239 | 0.045 | 12.5 | 12.2 | 19.2 | 3.6 | |
| 25 | 4.31 | 0.194 | 0.0004 | 0.025 | 23.1 | 0.190 | 0.282 | 0.034 | 13.6 | 14.0 | 20.7 | 2.5 | |
| 26 | 3.73 | 0.189 | 0.0003 | 0.013 | 13.1 | 0.106 | 0.103 | 0.038 | 8.6 | 12.2 | 12.0 | 4.4 | |
| 27 | 4.13 | 0.209 | 0.0003 | 0.025 | 20.0 | 0.162 | 0.199 | 0.018 | 13.1 | 12.4 | 15.2 | 1.4 | |
| Total. | 40.79 | 1.953 | 0.0028 | 0.222 | 171.5 | 1.409 | 1.815 | 0.427 | 107.7 | | | | |
| Average. | 4.08 | 0.195 | 0.0003 | 0.022 | 17.15 | 0.141 | 0.1815 | 0.043 | 10.77 | 13.1 | 16.85 | 4.0 | |
| 28 | 4.29 | 0.263 | 0.0004 | 0.022 | 25.6 | 0.214 | 0.343 | 0.054 | 15.7 | 13.6 | 21.9 | 3.4 | 1 cc. parathyroid extract at 12 noon and 7.45 p.m. |
| 29 | 3.81 | 0.355 | 0.0513 | 0.033 | 35.8 | 0.263 | 0.477 | 0.065 | 23.1 | 11.4 | 20.6 | 2.8 | 1 cc. parathyroid extract at 9.55 a.m. and 9.45 p.m. |
| 30 | 4.02 | 0.227 | 0.0304 | 0.003 | | | | | | | | | 1 cc. parathyroid extract at 9.45 a.m. and 10.45 p.m. |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| July | | | | | | | | | | | | | |
| 1 | 3.87 | 0.429 | 0.0736 | 0.034 | 30.6 | 0.263 | 0.477 | 0.049 | 18.6 | 14.1 | 25.6 | 2.6 | 1 cc. parathyroid extract at 10 a.m., 3.30, and 11.30 p.m. |
| 2 | 4.84 | 0.422 | 0.1754 | 0.044 | 30.3 | 0.272 | 0.536 | 0.084 | 17.7 | 15.3 | 30.2 | 4.7 | 1 cc. extract at 9.30 a.m., 2 cc. at 4 and 11.15 p.m. Dog refused to eat, fed with stomach tube. |
| 3 | | | | | 8.5 | 0.082 | 0.193 | 0.026 | 4.4 | 18.5 | 43.4 | 5.9 | Urine contaminated with blood, rejected. Dog died between 2 and 3 p.m., July 4. Hemorrhages into stomach and duodenum, none into colon. |
| Total. | 20.83 | 1.696 | 0.3311 | 0.136 | 130.8 | 1.094 | 2.026 | 0.278 | 79.5 | | | | |
| Average. | 4.17 | 0.339 | 0.0662 | 0.027 | 21.8 | 0.182 | 0.338 | 0.046 | 13.25 | 13.8 | 25.5 | 3.5 | |

TABLE III.

*Effect of Administration of Parathyroid Extract upon the Excretion of Nitrogen, Phosphorus, Calcium, and Magnesium.*

Dog 36. Female, weight 8.6 kilos. Fed daily a mixture of 20 gm. beef heart, 18 gm. cracker meal, 18 cc. maize oil, 10 gm. infusorial earth, and 400 cc. water. Additional water *ad libitum*.

| Date. | Urine. | | | | Feces. | | | | | | | | Remarks. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | Per gm. silica. | | | |
| | Nitrogen. | Phosphorus. | Calcium. | Magnesium. | Weight. | Phosphorus. | Calcium. | Magnesium. | Silica. | Phosphorus. | Calcium. | Magnesium. | |
| *1925* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *mg.* | *mg.* | *mg.* | |
| June | | | | | | | | | | | | | |
| 22 | 2.52 | 0.126 | 0.0002 | 0.015 | 9.0 | 0.066 | 0.118 | 0.029 | 5.53 | 12.0 | 21.3 | 5.3 | |
| 23 | 2.23 | 0.146 | 0.0001 | 0.016 | 14.8 | 0.124 | 0.189 | 0.043 | 8.80 | 14.1 | 21.5 | 4.9 | |
| 24 | 2.29 | 0.112 | 0.0001 | 0.019 | 13.4 | 0.102 | 0.149 | 0.048 | 8.90 | 11.4 | 16.7 | 5.4 | |
| 25 | 2.33 | 0.105 | 0.0085 | 0.011 | 17.1 | 0.119 | 0.189 | 0.060 | 10.48 | 11.4 | 18.1 | 5.7 | |
| 26 | 2.42 | 0.120 | 0.0087 | 0.004 | 11.2 | 0.096 | 0.130 | 0.031 | 7.01 | 13.8 | 18.6 | 4.4 | |
| 27 | 2.40 | 0.137 | 0.0040 | 0.007 | 12.6 | 0.111 | 0.108 | 0.044 | 7.48 | 14.9 | 14.4 | 5.9 | |
| Total. | 14.19 | 0.746 | 0.0216 | 0.072 | 78.1 | 0.618 | 0.883 | 0.255 | 48.20 | | | | |
| Average. | 2.365 | 0.124 | 0.0036 | 0.012 | 13.0 | 0.103 | 0.144 | 0.043 | 8.03 | 12.8 | 18.3 | 5.3 | |
| 28 | 2.80 | 0.213 | 0.0301 | 0.016 | 11.0 | 0.091 | 0.086 | 0.022 | 7.22 | 12.6 | 11.9 | 3.1 | 1 cc. parathyroid extract at noon and 7.45 p.m. |
| 29 | 2.21 | 0.164 | 0.0365 | 0.015 | 12.0 | 0.099 | 0.157 | 0.031 | 7.75 | 12.8 | 20.2 | 4.0 | 1 cc. parathyroid extract at 9.45 a.m. and 9.45 p.m. |
| 30 | 2.67 | 0.207 | 0.0355 | 0.021 | 4.2 | 0.026 | 0.042 | 0.012 | 2.75 | 9.6 | 15.5 | 4.3 | 1 cc. parathyroid extract at 9.45 a.m. and 10.45 p.m. |
| July | | | | | | | | | | | | | 1 cc. parathyroid extract at 10 |
| 1 | 3.89 | 0.420 | 0.1125 | 0.039 | 14.4 | 0.112 | 0.206 | 0.022 | 9.12 | 12.3 | 22.6 | 2.4 | a.m., 3.30 and 11.30 p.m. |
| 2 | 3.98 | 0.393 | 0.1010 | 0.028 | 8.3 | 0.077 | 0.126 | 0.016 | 4.65 | 16.6 | 27.1 | 3.5 | 1 cc. parathyroid extract at 9.30 a.m., 3.30 and 11.30 p.m. |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 3 | | | | | 15.9 | 0.125 | 0.204 | 0.048 | 8.66 | 14.4 | 23.6 | 5.5 | Refused food, fed by stomach tube, vomited. July 3, 10.45 a.m., serum contains 20.5 mg. Ca per 100 cc. |
| Total. | 15.55 | 1.397 | 0.3156 | 0.119 | 65.8 | 0.530 | 0.821 | 0.151 | 40.15 | | | | |
| Average. | 3.11 | 0.279 | 0.0631 | 0.024 | 11.0 | 0.088 | 0.137 | 0.025 | 6.69 | 13.2 | 20.4 | 3.8 | Dog found dead 8 a.m., July 4. Large intestine empty, no sign of hemorrhage. Stomach and duodenum full of blood, some of which had been vomited. |

the following period, there was a marked increase in the excretion of both phosphorus and calcium. This increase continued for several days and, even 11 days after the last injection, the excretion of calcium and phosphorus had not returned to its previous level.

The excretion of magnesium in the urine was unaffected by the administration of parathyroid extract but the excretion in the feces was increased.

In the following two experiments, urine and feces were analyzed daily. The administration of parathyroid extract was continued until symptoms of more severe intoxication resulted. Both dogs died. In the former of these experiments, the fore period was 10 days long and the results in Table II show very well the variations that may be expected, from day to day, in the composition of the feces. After the administration of the parathyroid extract, there was a prompt increase in the fecal excretion of calcium. The amount of phosphorus in the feces was increased to a lesser extent and only after 3 or 4 days. But examination of the data for the urinary excretion shows that the amount of phosphorus was increased by over 100 per cent, as against an increase of only 20 per cent in the amount of nitrogen. And the absolute increase in the amount of phosphorus, approximately 0.7 gm., was far more than the equivalent of the increase in the amount of calcium in the urine, which was 0.174 gm.

In this experiment, the excretion of magnesium in both urine and feces remained practically unchanged.

In the last experiment of this series, as in the first, the excretion of calcium in the feces had not reached a constant level when the administration of parathyroid extract was begun. The steady decrease during the fore period continued for another day but, thereafter, there was a pronounced increase in the amount of calcium excreted. The increase in the amount of phosphorus in the feces was much less marked. But, precisely as in the previous experiment, the amount of phosphorus in the urine was increased by over 120 per cent while the amount of nitrogen was increased by only 33 per cent. The absolute increase in the amount of phosphorus, 0.77 gm. was much greater than the equivalent of the increase in the amount of calcium in the urine, which was about 0.3 gm.

In this experiment, the amount of magnesium in the urine was increased, and the amount in the feces slightly increased, the total excretion remaining practically unchanged.

## DISCUSSION.

Holt, La Mer, and Chown (3) have recently shown that there is a marked reduction in the concentration of both calcium and phosphate in solution when blood serum is shaken with solid $Ca_3(PO_4)_2$. They concluded that serum is normally supersaturated with calcium phosphate and have postulated the presence, in serum, of a substance which delays the precipitation of calcium absorbed from the intestine. They make no attempt to explain how the calcium content of the blood is maintained at a normal level, in spite of a loss of calcium from the body, when the food contains no calcium or quite inadequate amounts.

Holt, La Mer, and Chown claim that there is no diminution in the ion product when blood serum is shaken in the absence of $Ca_3(PO_4)_2$ and that, therefore, the reduction on shaking with $Ca_3(PO_4)_2$ is due, not to a decreased solubility as a result of the shaking but is due to the acceleration of the equilibrium condition as a result of the presence of the solid phase. They present no experiments in which the serum was so treated without calcium phosphate but, assuming the correctness of the statement, it may merely mean that a hypothetical substance which keeps calcium phosphate in solution is more rapidly destroyed in the presence of the solid particles than in their absence.

It may be that there is one substance, in blood, that dissolves calcium phosphate from bone and another, in serum, that delays precipitation but the simpler hypothesis that there is only one substance appears to be preferable.

It is our belief that the parathyroid hormone is the substance that keeps calcium phosphate in solution, or, at least, that it is necessary to the preparation of this hypothetical substance. But our experiments seem to indicate that the substance does not merely retard the precipitation of calcium phosphate but that it actually dissolves it; whether by combination with calcium, phosphate, or both, it is impossible, at present, to determine. Our animals received practically no calcium in their food. Yet the calcium content of the blood was increased. This must have

been at the expense of certain tissues. Since the increased excretion of calcium was regularly accompanied by an approximately equivalent increase in the excretion of phosphorus, it is not unreasonable to assume that both calcium and phosphorus were derived from bone, particularly since there seems to be no other tissue that could supply the large amounts lost.

We believe that removal of the parathyroids leads to a prompt shortage of this hypothetical substance. Consequently, calcium phosphate is precipitated. . There is a prompt decrease in the excretion of phosphate (1, 4–6). The concentration of phosphate in the plasma is kept at its normal level or even above that (7–12) by the phosphate derived from the metabolism of proteins, phosphatides, etc., but the calcium content must fall unless calcium salts are administered. Large amounts of these are necessary because much of the calcium is precipitated as soon as it is absorbed. Unpublished experiments show that large quantities of calcium phosphate may be so deposited.

Excess of parathyroid secretion leads to excessive solution of calcium phosphate from the bones and, possibly, other tissues and to an increase in the concentration of both calcium and phosphate in the blood. The kidneys speedily remove the excess of the latter so that the increase may be too slight to be detected by our present methods (2, 13, 14). The increase in the concentration of calcium is much more evident. Both substances are excreted in increased amounts and in approximately equivalent proportions.

The implications of this hypothesis will be more fully discussed in a subsequent publication but we must here repeat and emphasize Collip's warning against any but the most careful and discriminating clinical use of parathyroid extract. It should be clearly understood by those who attempt to use it that the hypercalcemia it induces is not due to improved assimilation but is the cause of an increased loss of calcium. It is not to be used to promote calcium assimilation for only the contrary result is to be anticipated.

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# MINERAL METABOLISM OF THE LYMPH FOLLOWING INJECTIONS OF LEVO- AND DEXTRO-SUPRARENIN, PITUITRIN, AND PILOCARPINE.

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In a previous paper (1) dealing with alterations in the lymph following the injection of epinephrine we have demonstrated an evident change of the vascular endothelium whereby it becomes less permeable for a considerable period of time following the injections.

In a paper of this present series (2) we have also shown that in anaphylaxis, when the endothelium becomes suddenly permeable, ionic alterations take place which seem directly related to the alterations of the cellular metabolism that we might anticipate, that is a calcium increase in the lymph with a diminution of sodium and potassium (retained by the cells when they are stimulated?). Obviously the autonomic nervous system must be intimately connected with such alterations, probably as a parasympathetic overbalance (see the papers of Tinel and Santenoise (3), Schittenhelm (4), and Tonietti (5), etc.).

For such experiments the lymph offers certain advantages over blood analysis, which has been carried out by a number of observers.

Thus Dresel and Katz (6) have followed the potassium level in the serum after adrenalin injections and have found that for the first half hour potassium is diminished; this is followed by a period when it is increased. Billigheimer (7) found a diminution of calcium while Vollmer (8) has determined that a preliminary period of acidosis is followed by a period of relative alkalosis following adrenalin injections. He illustrates the diphasic relation of the ionic change in the manner shown in the chart. Leicher

(9), working with patients, states that he finds that adrenalin injections tend to lower the calcium level of the blood.

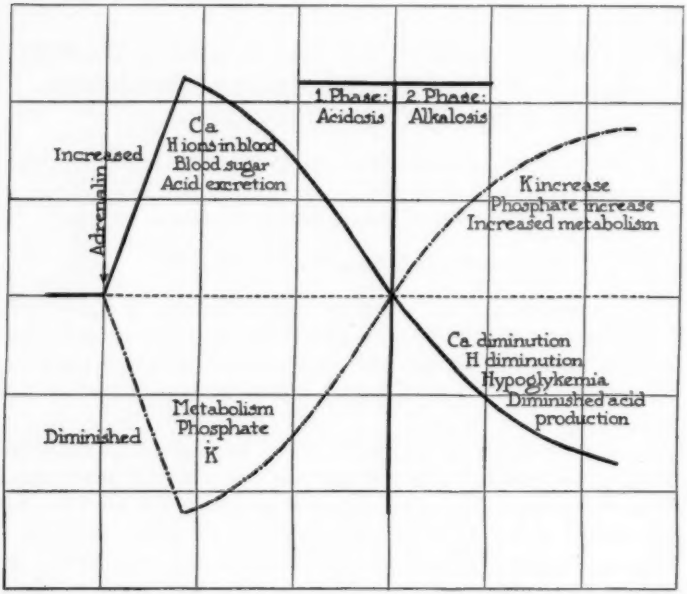


CHART 1. Vollmer's graphic presentation of the alterations following adrenalin injection.

## EXPERIMENTAL.[1]

### *Epinephrine.*

We have examined the effects of epinephrine injections on the mineral metabolism of the lymph in nine dogs; in two groups of

[1] The technique used has been similar to that previously described (1, 2). We have endeavored to use only quiet animals (the entire experiment being conducted under local anesthetic) but naturally considerable variation must be expected with the degree of nervous reaction of the animal. Particularly the sugar level of the lymph and the calcium content may show considerable fluctuation. Even in the blood of humans Glaser (10) has noted considerable variation in calcium with different psychic states and the blood level is naturally much more constant than the lymph.

three each the lymph has been pooled because the volume following adrenalin injections is so small that individual determinations are not possible.

The following protocols are appended.

(1) 5 cc. 1/10,000 adrenalin intravenously. (Sugar, $CO_2$-combining power, amino acids.)
(2) 2 cc. 1/10,000 adrenalin intravenously. Mineral metabolism.
(3) 10 mg. levo-suprarenin bitartrate (Metz) intravenously. Fatal dose. Mineral metabolism.

PROTOCOL 1.

Weight of dog 11 kilos. 5 cc. 1/10,000 adrenalin intravenously.

| Sample No. | Time. | Volume. | Volume per 5 min. | Amino acids. | $CO_2$ combining power. | Sugar. |
|---|---|---|---|---|---|---|
| | *min.* | *cc.* | *cc.* | *mg. per 100 cc.* | | *mg. per 100 cc.* |
| 1 | 15 | 7.5 | 2.5 | 2.45 | 34 | 100 |
| 2 | 15 | 6.3 | 2.1 | 2.33 | 36 | 115 |
| 3 | 15 | 4.2 | 1.4 | | 32 | |
| Injection of 5 cc. 1/10,000 adrenalin intravenously. | | | | | | |
| 4 | 5 | 2.0 | 2.0 | | 26 | |
| 5 | 5 | 3.8 | 3.8 | 2.16 | 30 | 118 |
| 6 | 5 | 2.2 | 2.2 | | 40 | |
| 7 | 15 | 6.7 | 2.3 | 4.16 | 52 | |
| 8 | 15 | 8.5 | 2.8 | 2.97 | | 129 |
| 9 | 15 | 6.5 | 2.2 | 3.84 | 28 | 133 |
| 10 | 15 | 7.3 | 2.4 | | 39 | 118 |
| 11 | 30 | 11.1 | 1.85 | 4.16 | 41 | 129 |
| 12 | 30 | 10.0 | 1.66 | 3.99 | | 133 |
| | *hr.* | | | | | |
| 13 | 1 | 18.0 | 1.5 | 3.16 | 38 | 112 |
| 14 | 1 | 18.5 | 1.5 | 2.93 | | 114 |
| | *min.* | | | | | 112 |
| 15 | 30 | 9.6 | 1.6 | | | |

(4) 2 mg. levo-suprarenin bitartrate (Metz) intravenously. Mineral metabolism. Pooled samples of three dogs.
(5) 2 mg. levo-suprarenin bitartrate (Metz) intravenously. Mineral metabolism. Pooled samples of three dogs.

The microanalysis for the various ions has been quite satisfactory with the exception of the potassium values. These we regard as doubtful but we have included the results in the protocols expressed in terms of an arbitrary standard for each experiment.

Protocol 1 reveals two of the alterations that Vollmer has brought out, *viz.* an *early acidosis* indicated by the reduction in the combining power of the lymph for the first 15 minutes, followed later by a relative alkalosis, and then a return to normal. Secondly, a primary reduction of metabolism indicated by the reduction in amino acid from the normal of 2.33 to 2.16, followed by an

PROTOCOL 2.

Weight of dog 20 kilos. 2 cc. 1/10,000 adrenalin intravenously.

| Sample No. | Time. | Volume. | Volume per 5 min. | Protein. | Fibrin. | Chlorides. | Phosphates. | Calcium oxide. | Magnesium. | Sodium. | Potassium (relative values). |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *min.* | *cc.* | *cc.* | *mg. per cc.* | *mg. per cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg per 100 cc.* | *mg. per 100 cc.* | |
| 1 | 15 | 9.2 | 3.1 | 48.4 | 1.74 | 770 | 3.57 | 19.3 | 2.90 | 171 | 0.80 |
| 2 | 15 | 8.2 | 2.7 | | 1.73 | 770 | 3.70 | 19.1 | 2.79 | 160 | 0.92 |
| Injection of 2 cc. 1/10,000 adrenalin intravenously. | | | | | | | | | | | |
| 3 | 5 | 5.7 | 5.7 | 45.4 | 2.26 | 770 | 3.84 | 17.5 | 2.52 | 162 | 0.92 |
| 4 | 5 | 6.7 | 6.7 | | | | | | | | |
| 5 | 5 | 4.5 | 4.5 | | | | | | | | |
| 6 | 5 | 3.3 | 3.3 | 40.7 | 2.37 | 770 | 3.44 | 18.8 | 2.67 | 171 | 0.80 |
| 7 | 5 | 2.8 | 2.8 | | | | | | | | |
| 8 | 15 | 6.5 | 2.2 | 41.2 | 1.46 | 790 | 3.22 | 17.3 | 2.74 | 153 | 1.04 |
| 9 | 15 | 7.2 | 2.4 | | | | | | | | |
| 10 | 15 | 5.0 | 1.7 | 50.8 | 1.45 | 790 | 3.44 | 18.2 | 2.52 | 126 | 1.11 |
| 11 | 15 | 6.7 | 2.2 | | | | | | | | |
| 12 | 15 | 8.4 | 2.8 | | 1.10 | 780 | 3.70 | 17.2 | 2.91 | 171 | 0.92 |
| 13 | 15 | 7.0 | 2.3 | | | | | | | | |
| 14 | 15 | 6.7 | 2.2 | 50.7 | 1.28 | 780 | 4.34 | 17.5 | 3.08 | 126 | 1.46 |
| 15 | 15 | 6.0 | 2.0 | | | | | | | | |
| 16 | 15 | 7.5 | | | | | | | | | |

increase that has persisted to the end of the experiment. In this particular experiment the typical effect in the reduction of the lymph flow (after the initial increase) is not apparent.

In this experiment, the calcium increase expected immediately after the injection is not observed, nor is there any increase in magnesium during the first 80 minutes following the injection.

The potassium values, with a delayed increase, and the phosphate values, are typical of the effects found by Vollmer.

This experiment reveals the marked alterations found in fatal intoxication. The results are quite the reverse of those described by Vollmer immediately after the injection, *i.e.* a diminution of calcium and magnesium, with an increase in potassium.

If we now examine the results of Protocols 4 and 5, representing

PROTOCOL 3.

Weight of dog 10 kilos. 10 mg. *l*-suprarenin bitartrate (Metz) intravenously. (Fatal in 3 hours.)

| Sample No. | Time. | Volume. | Volume per 5 min. | Protein. | Fibrin. | Phosphates. | Calcium oxide. | Sodium. | Magnesium. | Potassium (relative values). |
|---|---|---|---|---|---|---|---|---|---|---|
| | *min.* | *cc.* | *cc.* | *mg. per cc.* | *mg. per cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | |
| 1 | 15 | 10.8 | 3.6 | 65.3 | 1.29 | 3.33 | 17.5 | 232 | 3.57 | 0.45 |
| 2 | 15 | 10.8 | 3.6 | 69.2 | 0.91 | 3.12 | 16.4 | 248 | 2.43 | 0.57 |
| Injection of 10 mg. *l*-suprarenin bitartrate intravenously. | | | | | | | | | | |
| 3 | 5 | 1.1 | 1.1 | | | | | | | |
| 4 | 5 | 2.3 | 2.3 | 71.35 | 1.15 | | 16.5 | 259 | 2.01 | 1.80 |
| 5 | 5 | 2.7 | 2.7 | | | | | | | |
| 6 | 15 | 10.0 | 3.3 | 82.2 | 2.6 | 3.44 | 15.9 | 212 | 2.35 | 0.26 |
| 7 | 15 | 9.1 | 3.0 | 81.14 | 1.54 | 6.42 | 18.3 | 364 | 3.23 | 0.20 |
| 8 | 15 | 14.5 | 4.8 | 66.26 | 1.26 | 7.14 | 14.7 | 248 | 2.56 | 0.25 |
| 9 | 15 | 10.3 | 3.4 | 78.25 | 1.25 | 6.66 | 14.8 | 248 | 2.94 | 0.28 |
| 10 | 15 | 7.7 | 2.6 | | | | | | | |
| 11 | 15 | 4.0 | 1.3 | 45.7 | 1.28 | 5.27 | 16.1 | 248 | 2.94 | 0.26 |
| 12 | | 7.0 | 2.3 | | | | | | | |
| Serum (end of experiment) | | | | 119.0 | | 3.33 | 17.6 | 212 | 2.95 | 0.21 |

six dogs, we find the results more nearly in harmony with the figures of Vollmer. If we average the volume of lymph we observe first of all the typical effect:

Before injection, average for 5 minutes.....1.93
1st 5 min. after injection.....2.33
2nd 5 " " " .....3.3
3rd 5 " " " .....3.0

| | |
|---|---|
| 15 min. period | 1.8 |
| 15 " " | 1.45 |
| 15 " " | 1.39 |
| 30 " " | 1.3 |
| 30 " " | 1.5 |

*i.e.*, a primary increase in flow, followed by a distinct retardation. The calcium and phosphates of the lymph average as follows:

| | Calcium. | Phosphates. |
|---|---|---|
| Before | 10.4 | 2.07 |
| 15 min. after | 10.6 | 2.08 |
| 15 " " | 10.7 | 1.89 |
| 15 " " | 12.1 | 1.86 |
| 15 " " | 10.8 | 2.04 |
| 30 " " | 10.7 | 1.92 |
| 30 " " | 11.5 | 2.1 |
| 30 " " | | 2.5 |

*i.e.*, a slight primary increase in calcium with a primary reduction of phosphates, followed by a period when the calcium obviously fluctuates while the phosphates increase. Examination of the protocols makes it apparent that the potassium-calcium ratio is altered to the potassium side even when there is seemingly little change in the actual calcium figures.

While the experimental conditions and dosages employed have differed considerably from those of Vollmer, who worked with small subcutaneous doses in humans, our results give some evidence of the diphasic effect to which he has called attention.[2] A transient diminution of the phosphate level is followed by a striking increase, an acidosis is followed by a relative alkalosis. Other alterations in the mineral balance seem less constant. A primary reduction in the potassium level has not been apparent in our experiments, indeed in the animal that received the fatal dose (Protocol 3) a striking increase followed immediately on the injection. Magnesium showed the most marked changes, being usually increased, with a primary diminution apparent in some of the experiments.

[2] Zondek and Ucko (11) have extended the observation of such diphasic effects to all the endocrine reactions.

Since the fluctuations in the level of the mineral constituents of the lymph are much greater than they are in the blood, the variations encountered offer greater difficulty in interpretation. Boothby and Rowntree (12) have shown that the calorigenic action of adrenalin is at its maximum within a few minutes after

PROTOCOL 4.

Pooled samples, three dogs, weights respectively 15, 83, and 15 kilos. 2 mg. levo-suprarenin bitartrate intravenously.

| Sample No. | Dog 1. | Dog 2. | Dog 3. | Time. | Volume per 5 min. | Protein. | Fibrin. | Phosphates. | Calcium oxide. | Sodium | Magnesium. | Potassium (relative values). | Chlorides. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *min.* | *cc.* | *mg. per cc.* | *mg. per cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | | *mg. per 100 cc.* |
| 1 | 7.2 | 9.4 | 7.8 | 15 | 2.7 | Not analyzed. | | | | | | | |
| 2 | 5.7 | 9.4 | 6.0 | 15 | 2.3 | 40.2 | 1.8 | 2.35 | 15.0 | 227 | 1.94 | 0.62 | 600 |
| Injection of 2 mg. levo-suprarenin bitartrate intravenously. | | | | | | | | | | | | | |
| 3 | 2.3 | 3.3 | 4.5 | 5 | 3.37 | | | | | | | | |
| 4 | 3.8 | 4.5 | 5.4 | 5 | 4.6 | 40.1 | 2.14 | 2.5 | 15.0 | 171 | 1.93 | 0.76 | 558 |
| 5 | 5.1 | 3.1 | 3.2 | 5 | 3.8 | | | | | | | | |
| 6 | 7.6 | 5.0 | 6.2 | 15 | 2.1 | 37.7 | 1.81 | 2.06 | 15.15 | 184 | 2.36 | 0.66 | 540 |
| 7 | 3.4 | 5.5 | 4.2 | 15 | 1.4 | 40.6 | 1.76 | 1.97 | 16.6 | 167 | 2.24 | 0.55 | 540 |
| 8 | 1.4 | 4.3 | 5.0 | 15 | 1.17 | 40.6 | 3.00 | 2.42 | 16.3 | 244 | 2.17 | 0.71 | |
| 9 | 2.0 | 4.3 | 4.6 | 15 | 1.2 | 35.8 | 1.72 | 2.08 | 14.85 | 222 | 2.47 | 1.05 | 590 |
| 10 | 1.8 | 4.5 | 6.2 | 15 | 1.4 | | | | | | | | |
| 11 | 3.4 | 4.5 | 5.0 | 15 | 1.43 | 38.7 | 1.62 | 2.2 | 16.6 | 211 | 2.19 | 0.91 | 585 |
| 12 | 5.0 | 5.6 | 7.0 | 15 | 1.95 | | | | | | | | |
| 13 | 5.1 | 4.6 | 4.6 | 15 | 1.6 | 36.7 | 1.72 | 3.0 | 16.4 | 211 | 1.88 | 0.71 | 575 |
| 14 | 6.0 | 7.4 | 7.4 | 15 | 2.3 | | | | | | | | |
| 15 | 6.0 | 7.0 | 4.7 | 15 | 2.0 | 39.3 | 1.8 | 3.41 | 15.5 | 211 | 1.8 | 0.65 | 565 |
| 16 | 7.6 | 6.4 | 4.1 | 15 | 2.0 | | | | | | | | |
| 17 | 7.6 | 5.4 | 5.6 | 15 | 2.06 | 36.3 | 1.62 | 3.1 | 15.35 | 289 | 2.08 | 0.71 | 535 |
| 18 | 6.5 | 7.4 | 3.6 | 15 | 2.0 | | | | | | | | |

administration. Probably much of this effect will take place in the liver, where Kornfeld and Elias (13) assume that a local acidity may develop. Gottschalk and Pohle (14) have demonstrated this by actual observation and it has also been shown that the administration of alkali will prevent the hyperglycemia after

adrenalin injection. The acidosis, apparently from partially oxidized metabolites, results from the increased metabolism of the liver with diminished oxygen supply.

Associated with the calorigenic action of adrenalin in increase in oxygen consumption and $CO_2$ output has been determined by Boothby and by Lyon (15). But on the isolated cell a reduction in the $CO_2$ output has been noted by Hutchinson (16) (Muscle

PROTOCOL 5.

Pooled samples, three dogs, weights respectively 8, 7, and 12 kilos. Each animal received 2.5 cc. 1/50,000 adrenalin intravenously.

| Sample No. | Time. | Dog 1. | Dog 2. | Dog 3. | Volume. | Volume per 5 min. per dog. | Protein. | Fibrin. | Chlorides. | Phosphates. | Calcium oxide. | Magnesium. | Potassium (relative values). |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *min.* | | | | *cc.* | *cc.* | *mg. per cc.* | *mg. per cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc* | *mg. per 100 cc.* | |
| 1 | 15 | 4.4 | 4.3 | 7.7 | | | | | | | | | |
| 2 | 15 | 3.3 | 4.2 | 6.5 | 42.3 | 1.56 | 42.7 | 1.3 | 670 | 1.79 | 14.2 | 4.35 | 0.313 |
| 3 | 15 | 3.6 | 3.1 | 5.1 | | | | | | | | | |
| Injection of 2.5 cc. 1/50,000 adrenalin intravenously. | | | | | | | | | | | | | |
| 4 | 5 | 1.0 | 1.4 | 1.5 | | 1.3 | | | | | | | |
| 5 | 5 | 2.5 | 1.6 | 2.1 | 16.5 | 2.1 | 41.8 | 1.0 | 665 | 1.66 | | | 0.435 |
| 6 | 5 | 1.7 | 1.6 | 3.1 | | 2.1 | | | | | | | |
| 7 | 15 | 4.7 | 3.4 | 5.5 | 13.6 | 1.5 | 44.0 | 0.9 | 697 | 1.73 | 14.9 | 4.0 | 0.625 |
| 8 | 15 | 3.7 | 3.8 | 5.9 | 13.4 | 1.5 | 42.4 | 1.0 | 670 | 1.75 | 17.3 | 3.16 | 0.526 |
| 9 | 15 | 3.6 | 3.8 | 7.2 | 14.6 | 1.6 | 43.9 | 0.3 | 660 | 1.66 | 14.2 | 3.91 | 0.400 |
| 10 | 15 | 3.3 | 2.6 | 6.1 | 12.0 | 1.3 | 43.2 | 0.6 | 670 | 1.75 | 15.0 | 3.93 | 0.555 |
| 11 | 15 | 3.0 | 2.6 | 5.8 | 11.4 | 1.3 | 42.7 | 0.7 | 675 | 1.79 | 15.2 | 4.54 | 0.465 |
| 12 | 15 | 2.5 | 2.8 | 6.0 | 11.3 | 1.3 | 40.1 | 0.8 | 665 | 1.93 | 14.7 | 4.96 | 0.487 |
| 13 | 15 | 2.7 | 3.1 | 6.0 | 11.8 | 1.3 | 41.6 | 0.8 | 655 | 2.09 | 16.8 | 5.97 | 0.667 |
| 14 | 15 | 3.0 | 3.3 | 5.8 | 12.1 | 1.3 | 39.4 | 0.7 | 635 | 2.00 | 16.9 | 5.48 | 0.416 |

and radish seedling). Lange (17) has shown that the cell membrane has a diminished permeability and that in muscle the excretion of phosphoric acid from the cell and the entrance of potassium into the cell is diminished by adrenalin. This has been confirmed by Okamoto (18). Freund and Janssen (19) have finally been able to show that such alterations in the mineral metabolism of the muscle may take place irrespective of contraction.

We can hardly expect that alterations will make their appearance in any definite sequence in the lymph. Disturbances of the vascularization of the liver follow the injections, and the collected lymph will reflect such alterations as well as the effects of the adrenalin on the individual cell. In addition we have the physical effect of a sudden augmentation of the lymph volume as a result of the ultrafiltration of much fluid from the blood. Preexisting inequalities in the level of the various ions in the blood and lymph will then result in a sudden shifting of the lymph values for such

PROTOCOL 6.

Weight of dog 10 kilos. 10 mg. dextro-suprarenin bitartrate intravenously.

| Sample No. | Time. | Volume of lymph. | Volume per 5 min. | Phosphates. | Calcium oxide. | Magnesium. | Sodium. | Potassium (relative values). | Sugar. |
|---|---|---|---|---|---|---|---|---|---|
| | *min* | *cc.* | *cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | | *mg. per 100 cc.* |
| 1 | 15 | 12.0 | 3.0 | 1.81 | 20.3 | 3.5 | 265 | 100 | 152 |
| Injection of 10 mg. *d*-suprarenin bitartrate. | | | | | | | | | |
| 2 | 5 | 2.5 | 2.5 | | | | | | |
| 3 | 5 | 2.0 | 2.0 | | 21.4 | | | | 179 |
| 4 | 5 | 3.0 | 3.0 | | | | | | |
| 5 | 15 | 7.2 | 2.4 | 1.66 | 22.0 | 4.0 | 278 | 77 | 164 |
| 6 | 15 | 8.1 | 2.7 | 1.66 | 22.6 | 4.0 | 322 | 90 | 164 |
| 7 | 15 | 7.5 | 2.5 | 1.66 | 23.0 | 4.0 | 278 | 87 | 160 |
| 8 | 15 | 8.2 | 2.7 | 2.07 | 19.3 | 4.36 | 266 | 73 | 164.5 |
| 9 | 15 | 9.4 | 3.1 | 2.22 | 19.5 | | 288 | 90 | 175 |
| 10 | 15 | 10.0 | 3.3 | 1.77 | 19.7 | 4.36 | 288 | 77 | 155 |
| 11 | 15 | 8.5 | 2.8 | 1.94 | 21.2 | 4.4 | 278 | 83 | |
| 12 | 15 | 6.0 | 2.0 | 1.87 | 18.7 | 5.0 | 288 | 140 | |

ions. Nor can we assume that the hormone itself acts purely as a sympathetic stimulant. Much evidence has recently been presented indicating that adrenalin actually effects both the nerve endings of the para-sympathetic as well as the sympathetic system, and that the relative response will depend largely on the concentration of ions present about the cell at the particular moment. The papers of Dresel (6), of Baráth (20), and of Kylin and Nyström (21) are of particular interest in this connection.

*Dextro-Suprarenin Bitartrate.*

Considering that many of the alterations of lymph composition observed following levo-suprarenin injection, must take origin in liver alterations associated with the pronounced engorgement of that organ and a concomitant acidosis, we have been interested to observe whether dextro-suprarenin, relatively inert in so far as pressor effect is concerned, would reveal alterations comparable

PROTOCOL 7.

Pooled samples, three dogs, weights respectively 8, 9, and 11 kilos. Each animal received 2 mg. dextro-suprarenin bitartrate (Metz) intravenously.

| Sample No. | Time. | Volume. 3 dogs. | Volume per 5 min. per dog. | Protein. | Fibrin. | Sugar. | Chlorides. | Phosphates. | Calcium oxide. | Magnesium. | Potassium (relative value). | Sodium. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *min.* | *cc.* | *cc.* | *mg. per cc.* | *mg. per cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg per 100 cc.* | *mg. per 100 cc.* | | *mg. per 100 cc.* |
| 1 | 15 | 17.9 | 2.0 | 54.4 | 1.62 | 85 | 550 | 1.92 | 16.4 | 2.61 | 1.00 | 222 |
| Injection of 2 mg. *d*-suprarenin bitartrate. | | | | | | | | | | | | |
| 2 | 5 | 6.4 | 2.1 | | | | | | | | | |
| 3 | 5 | 6.8 | 2.2 | 51.7 | 1.77 | 105 | 550 | 1.92 | 17.3 | 2.80 | 1.30 | 211 |
| 4 | 5 | 8.3 | 2.7 | | | | | | | | | |
| 5 | 15 | 17.9 | 2.0 | 49.04 | 1.84 | 107 | 600 | 1.92 | 17.7 | 2.80 | (0.75) | 144 |
| 6 | 15 | 15.7 | 1.7 | 55.12 | 1.72 | 106 | 560 | 1.76 | | | 1.30 | 145 |
| 7 | 15 | 11.6 | 1.3 | 51.55 | 1.55 | 106 | 550 | 2.00 | 18.4 | 2.61 | 1.00 | 200 |
| 8 | 15 | 8.7 | 0.9 | 51.59 | 1.59 | 93 | 495 | 1.81 | 15.8 | 2.61 | 1.36 | |
| 9 | 15 | 14.5 | 1.6 | | | | | | | | | |
| 10 | 15 | 11.2 | 1.2 | 45.94 | 1.34 | 97 | 410 | 1.92 | 17.9 | 2.61 | 0.80 | |
| 11 | 15 | 12.6 | 1.4 | | | | | | | | | |

to some of the effects of the active substance.[3] The following protocols show the effects of a single large dose (10 mg.) and the analysis of pooled samples of three dogs which received 2 mg. each.

It will be observed in Protocol 6 that there is a reduction in lymph flow immediately on injection, with a return to the normal level about an hour later. The effect on phosphates as well as

[3] Through the kindness of Mr. Metz we have been supplied with the *d*-suprarenin which has been used in these experiments.

on the calcium is typical of the Vollmer curve; this is true of the potassium as well, in that it shows a primary decrease and a later recovery. The continued increase in magnesium is of interest.

In the pooled samples with a smaller dose (Protocol 7) an increase in volume becomes apparent after 10 minutes; it is followed by the reduction in volume that is typical for *l*-suprarenin. This persists for about 1 hour. With it is associated a

PROTOCOL 8.

Pooled samples, three dogs. Each animal received ½ cc. pituitrin (Armour) intravenously.

| Sample No. | Time. | Volume. 3 dogs. | Volume per 5 min. per dog | Protein. | Fibrin. | Sugar. | Phosphates. | Chlorides. | Calcium oxide. | Magnesium. | Potassium (relative value). | Sodium. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *min.* | *cc.* | *cc.* | *mg. per cc.* | *mg. per cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | | *mg. per 100 cc.* |
| 1 | 15 | 18.5 | 2.05 | 48.4 | 2.4 | 115 | 2.70 | 630 | 12.6 | 1.24 | 1.00 | 144 |
| 2 | 15 | 19.1 | 2.1 | 48.5 | 2.4 | 112 | 2.85 | 630 | 11.5 | 1.14 | 0.80 | 121 |
| Injection of ½ cc. pituitrin intravenously. | | | | | | | | | | | | |
| 3 | 5 | 2.70 | 0.9 | | | | | | | | | |
| 4 | 5 | 4.85 | 1.6 | | 2.0 | 115 | 3.33 | 640 | 13.8 | 1.05 | 0.52 | 155 |
| 5 | 5 | 5.3 | 1.7 | | | | | | | | | |
| 6 | 15 | 11.2 | 1.23 | 49.7 | 2.0 | 112 | 3.18 | 637 | 13.4 | 2.16 | 0.52 | 166 |
| 7 | 15 | 9.3 | 1.03 | 48.9 | 1.2 | | 3.73 | | 12.8 | 1.54 | 0.55 | 110 |
| 8 | 15 | 13.8 | 1.53 | 42.7 | 1.3 | | 3.33 | 640 | 11.6 | 1.54 | | 99.7 |
| 9 | 15 | 14.9 | 1.65 | 47.0 | 1.2 | 124 | 3.18 | 640 | 12.1 | 1.75 | 0.50 | 99 |
| 10 | 15 | 17.8 | 1.98 | | 1.23 | 128 | 3.00 | 630 | 13.1 | 1.64 | 0.50 | 110.8 |
| 11 | 15 | 16.7 | 1.85 | 46.8 | 1.3 | 133 | 3.00 | 667 | 10.7 | 1.87 | 0.40 | 199 |
| 12 | 15 | 14.5 | 1.60 | 47.6 | 1.8 | 140 | 2.94 | 655 | 10.9 | | 0.40 | 243 |

reduction of protein concentration of the lymph. Hyperglycemia occurs even before there is any change in the phosphate level. The magnesium revealed only a slight increase, in contradistinction to the previous experiment; the sodium diminished while the potassium increased (values irregular).

In general the alterations were similar to those following the injection of *l*-suprarenin.

*Pituitrin.*

With pituitrin, a number of rather striking alterations become apparent.

Three dogs were used in this experiment and the lymph samples pooled in order to obtain sufficient material for examination.

For the first 2 or 3 minutes after the injection the lymph flow ceases, then the flow is reduced and remains at a lower level for practically an hour after the injection, without great alteration

PROTOCOL 9.

Weight of dog 15 kilos. 12 mg. pilocarpine intravenously.

| Sample No. | Time. | Volume. | Volume per 5 min. | Protein. | Fibrin. | Chlorides. | Phosphates. | Calcium oxide. | Sodium. | Magnesium. | Potassium (relative values). |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *min.* | *cc.* | *cc.* | *mg. per cc.* | *mg. per cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | |
| 1 | 15 | 5.0 | 1.7 | | | | | | | | |
| 2 | 15 | 4.8 | 1.6 | 36.0 | 1.49 | 690 | 3.00 | 15.4 | 171 | 2.26 | 0.76 |
| 3 | 15 | 3.6 | 1.2 | | | | | | | | |
| Injection of 12 mg. pilocarpine intravenously. | | | | | | | | | | | |
| 4 | 15 | 5.0 | 1.7 | 35.6 | 1.10 | 680 | 4.22 | 15.6 | 180 | 2.16 | 0.86 |
| 5 | 15 | 5.0 | 1.7 | | | | | | | | |
| 6 | 15 | 5.3 | 1.8 | 37.1 | 1.26 | 720 | 3.15 | 15.9 | 171 | 2.46 | 0.70 |
| 7 | 15 | 5.0 | 1.7 | | | | | | | | |
| 8 | 30 | 11.5 | 1.9 | 45.9 | 1.65 | 690 | 3.45 | 20.5 | 162 | 2.62 | 0.60 |
| 9 | 30 | 11.2 | 1.8 | 34.5 | 1.18 | 680 | 3.50 | 16.5 | 189 | | 0.50 |
| 10 | 30 | 7.7 | 1.3 | | 1.81 | 720 | 2.69 | 16.7 | 216 | | 0.40 |

in the concentration of the protein. Sugar is unaltered until toward the end of the experiment, while the phosphates reveal a marked increase, quite the reverse of that observed after both *l*- and *d*-suprarenin.

Calcium is slightly increased. A striking mobilization of sodium becomes apparent at the end of the experiment, as well as immediately after the injection. Bayley, Davis, Whitman, and Scott (22) recently observed the reduction in lymph flow following pituitrin injection.

*Pilocarpine.*

The effect of pilocarpine on the lymph flow is well known, the capillaries becoming more permeable and the lymph becoming richer in protein as the permeability is increased. It has recently been suggested that the effect of the pilocarpine in small doses is

PROTOCOL 10.

Pooled samples, two dogs, weights 13 and 8 kilos. 10 mg. pilocarpine intravenously.

| Sample No. | Time. | Dog 1. | Dog 2. | Volume per 5 min. per dog. | Protein. | Fibrin. | Chlorides. | Phosphates. | Calcium oxide. | Magnesium. | Sodium. | Potassium (relative values). |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *min.* | | | *cc.* | *mg. per cc.* | *mg. per cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | |
| 1 | 15 | 5.2 | 4.0 | | | | | | | | | |
| 2 | 15 | 5.0 | 5.1 | 1.45 | 50.0 | 1.41 | 702 | 3.12 | 20.05 | 3.49 | 360 | 1.51 |
| 3 | 15 | 3.8 | 3.2 | | | | | | | | | |
| Injection of 10 mg. pilocarpine intravenously. | | | | | | | | | | | | |
| 4 | 5 | 2.0 | 2.5 | 2.25 | | | | | | | | |
| 5 | 5 | 2.4 | 4.3 | 3.35 | 53.9 | 1.83 | 672 | 3.45 | 19.65 | 3.60 | | 0.60 |
| 6 | 5 | 3.3 | 3.5 | 3.40 | | | | | | | | |
| 7 | 15 | 7.0 | 9.0 | 2.65 | 65.0 | 2.50 | 682 | 3.03 | 19.25 | 3.38 | 160 | 0.55 |
| 8 | 15 | 6.8 | 10.0 | 2.80 | 71.5 | 3.09 | 672 | 3.12 | 21.35 | 2.86 | 250 | 0.55 |
| 9 | 15 | 7.0 | 6.6 | 2.20 | 70.0 | 2.49 | 685 | 3.45 | 19.65 | 2.90 | 260 | 0.48 |
| 10 | 15 | 8.0 | 5.5 | | | | | | | | | |
| 11 | 15 | 8.0 | 5.0 | 2.10 | 61.9 | 2.10 | 675 | 3.12 | 20.30 | 2.95 | 260 | 0.39 |
| 12 | 15 | 7.6 | 4.6 | | | | | | | | | |
| 13 | 15 | 8.6 | 4.0 | 1.95 | 61.13 | 2.13 | 685 | 3.12 | 20.30 | 3.08 | 220 | 0.45 |
| 14 | 15 | 8.2 | 3.1 | | | | | | | | | |
| 15 | 15 | 8.8 | 2.3 | 1.90 | 60.54 | 2.14 | 705 | 3.22 | 20.30 | 2.91 | | 0.50 |
| 16 | 15 | 8.5 | 3.3 | | | | | | | | | |
| 17 | 15 | 6.2 | 2.8 | | | | | | | | | |
| 18 | 15 | 5.8 | 3.0 | 1.65 | 57.63 | 1.83 | 705 | 3.33 | 20.30 | 3.49 | 300 | 0.58 |
| 19 | 15 | 5.8 | 3.7 | | | | | | | | | |

first manifested directly on the nerve endings of the endothelial cells themselves, and that this stimulation accounts for the effect on the lymph flow. With larger doses one may have stimulation of the adventitial Rouget or smooth muscle cells, contraction

of the capillaries, and reduction of the flow. There is considerable evidence for this sequence (Nelson and Keiper (23)).

The protocols are selected to show differences in the reaction

PROTOCOL 11.

Weight of dog 20 kilos. Pilocarpine effect following preliminary calcium infection.

| Sample No. | Time. | Volume of lymph. | Volume per 5 min. | Protein. | Fibrin. | Chlorides. | Phosphates. | Calcium oxide. |
|---|---|---|---|---|---|---|---|---|
| | *min.* | *cc.* | *cc.* | *mg. per cc.* | *mg. per cc.* | *mg. per 100 cc.* | *mg. per. 100 cc.* | *mg. per 100 cc.* |
| 1 | 15 | 7.8 | 2.6 | 44.6 | 0.88 | 690 | 2.17 | 18.1 |
| 2 | 15 | 5.8 | 1.9 | 39.2 | 1.08 | 645 | 1.78 | 17.4 |
| 3 | 15 | 3.4 | 1.1 | 36.6 | 1.46 | | 1.72 | 19.8 |
| Injection of calcium (10 cc.), 10 per cent lactate. | | | | | | | | |
| 4 | 5 | 1.2 | 1.2 | | | | | |
| 5 | 5 | 2.2 | 2.2 | 45.0 | 1.97 | | 1.75 | 20.25 |
| 6 | 5 | 2.4 | 2.4 | | | | | |
| 7 | 15 | 4.8 | 1.6 | 45.4 | 1.62 | 682 | 2.52 | 30.0 |
| 8 | 15 | 3.6 | 1.2 | | | | | |
| 9 | 15 | 3.0 | 1.0 | 44.7 | 1.55 | | 2.87 | 23.9 |
| 10 | 15 | 2.4 | 0.8 | | | | | |
| Injection of 20 mg. pilocarpine intravenously. | | | | | | | | |
| 11 | 5 | 6.8 | 6.8 | 44.7 | 1.79 | 685 | 3.27 | 26.3 |
| 12 | 5 | 8.0 | 8.0 | 48.7 | 2.33 | 670 | 2.98 | 28.2 |
| 13 | 5 | 8.0 | 8.0 | 52.8 | 3.12 | 620 | 2.79 | 25.7 |
| 14 | 15 | 27.0 | 9.0 | 62.0 | 4.00 | 642 | 2.57 | 24.7 |
| 15 | 15 | 30.0 | 10.0 | 63.3 | 3.47 | 640 | 2.56 | 26.1 |
| 16 | 15 | 28.0 | 9.3 | 64.6 | 3.23 | 650 | 2.68 | 24.3 |
| 17 | 15 | 28.4 | 9.5 | 65.4 | 3.91 | 655 | 2.71 | 23.5 |
| 18 | 15 | 23.0 | 7.7 | 54.0 | 3.13 | 650 | 2.27 | 24.7 |
| 19 | 15 | 19.0 | 6.3 | 66.0 | 3.43 | 667 | 1.93 | 25.6 |
| 20 | 15 | 17.4 | 5.3 | 65.4 | 3.25 | 667 | 1.82 | 22.5 |
| 21 | 15 | 14.6 | 4.7 | 65.5 | 3.20 | 660 | 1.90 | |
| 22 | 15 | 15.4 | 5.1 | 65.6 | 3.31 | 692 | 1.78 | 26.5 |
| 23 | 15 | 6.4 | 3.2 | 57.8 | 1.68 | | 2.11 | |

of the animals. In the first animal (Protocol 9) the effect on the lymph flow, as well as on concentration, was relatively slight. Phosphates were strikingly increased immediately after the injection; a calcium increase was apparent during the only time

that the protein concentration was augmented to any degree and when the sodium reached its lowest concentration. The other alterations were relatively slight in character.

Protocol 10 gives the results in a pooled experiment of two dogs. In these animals the lymph flow and protein and fibrin concentration was altered to a much greater extent, while the effect on the phosphates and calcium was relatively small. A reduction in sodium and potassium is, however, evidently closely related to the injection. Here again the maximum protein and fibrin increase is associated with higher calcium (Sample 8) and with lowest sodium values (Sample 7).

Just as we have tried the effect of a preliminary sensitization with calcium in anaphylaxis (2) we have made a similar experiment (Protocol 11) preceding a pilocarpine injection. The animal (weight 20 kilos) was highly nervous at the beginning of the experiment, with rapid pulse and respiration. A total of 20 cc. of calcium lactate solution (10 per cent) was injected intravenously after a preliminary observation period of 45 minutes. 20 mg. of pilocarpine were then injected. The animal died 2½ hours later, the lymph containing much bile pigment.

A number of alterations are to be observed. The calcium injection was followed by an effect on the lymph flow and protein concentration, both increasing for some time, then being reduced to the normal. Calcium increase in the lymph does not, apparently, take place immediately, but begins at the end of the first 15 minute period and reaches its maximum in the following 30 minutes.

When pilocarpine is now injected, the lymphagogue effect and the effect on protein concentration are very pronounced (relative dose similar to previous experiments). There is a well marked increase in calcium and in phosphates. The chlorides are diminished.

*Summary of Alterations.*

*Levo- and Dextro-Suprarenin.*

| | |
|---|---|
| Volume | First increased, later diminished. |
| Protein | Diminished. |
| Fibrin | Increased. |
| Sugar | First increased, later diminished. |
| Phosphates | " diminished, " increased. |

| | |
|---|---|
| Calcium | Slight increase, generally diminished. |
| Magnesium | Varies. |
| Chlorides | Unaltered, occasionally diminished later. |
| Sodium | Diminished. |
| Potassium | Increased. |

*Pituitrin.*

| | |
|---|---|
| Volume | Diminished. |
| Protein | Unaltered. |
| Fibrin | Diminished. |
| Sugar | Late increase. |
| Phosphates | Increased. |
| Calcium | First increased, later diminished. |
| Magnesium | " " |
| Clorides | Late increase. |
| Sodium | " " (irregular). |
| Potassium | Increased. |

*Pilocarpine.*

| | |
|---|---|
| Volume | Increased. |
| Protein | " |
| Fibrin | " |
| Sugar | Diminished. |
| Phosphates | Increased. |
| Calcium | " |
| Magnesium | Irregular. |
| Chlorides | Diminished. |
| Sodium | " |
| Potassium | Increased. |

DISCUSSION.

Zondek and his associates as well as a number of other workers have attempted to demonstrate that differences in cellular reaction initiated by the autonomic nervous system are really brought about by means of changes taking place in the ionic equilibrium at the cell surface. The ions represent the tools used by the nervous mechanism. This seems reasonable since we assume that any alteration in the functional state of the cell is brought about by ionic alterations at interfaces. The direct chemical regulation is the most primitive method at the disposal of the organism.

In the schematic visualization of the autonomic nervous system heretofore popular with the pharmacologist and the physiologist it has been assumed that an autonomic impulse to the cell is

usually either sympathetic or para-sympathetic. Such a condition would be most satisfactory theoretically, but evidence has been accumulating that makes the basis unsafe. A like impulse may bring about quite unlike phenomena of stimulation, the result depending probably on the ionic equilibrium, existing at the moment in the tissue. Thus small doses of adrenalin may either cause hyperglycemia or hypoglycemia (Kylin), vasoconstriction or vasodilation (Collip (24), Bergengren (25)), depending on pre-existing local conditions.

But if the effect of autonomic response varies with local conditions ($H^+$ ion concentration, ion balance, etc.) the local ionic balance may presumably be disturbed by alterations in the nervous impulses. In vagotonia, Dresel, for instance, assumes that the tissues are more alkaline and contain less colloidal calcium and more potassium; the blood is also more alkaline, has little potassium, little ionized calcium, and more total calcium. In sympathicotonia these alterations are presumably reversed. We must, however, consider the possibility proposed by Lawaczeck (26) that in the mechanism of the nerve action an actual transfer of calcium ions, from cell to fluid or *vice versa*, need not take place. A mere shifting of indifferent, bound ions to the free ionic condition in and about the cell surface would have the same effect; a shifting that could take place readily enough with minor changes in $H^+$ ion concentration. Günther and Heubner (27), working with perfused frogs' hearts find, however, that both ionized as well as unionized calcium has a specific action. We mention the possibility however because it is well to keep in mind that the gross alterations that we may find in the lymph need by no means give us an actual picture of alterations that we might expect in the tissues. But quite apart from the specific effect on the autonomic nervous system with which we usually associate the agents that we have studied (adrenalin, pituitrin, and pilocarpine) we must not overlook the fact that these substances all have a direct effect on the isolated cell and that presumably they may directly act on tissues irrespective of autonomic nerve endings. Indeed, if we correctly interpret our results it is this effect which finds expression most clearly in the ionic alterations which we have recorded.

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# THE CONCENTRATION OF CHLORIDES IN THE GLOMERULAR URINE OF FROGS.*

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The interest and importance which will attach to reliable quantitative data concerning the composition of the fluid which is separated from the blood during its passage through the glomerular capillaries are obvious. Until such information shall be supplied, our knowledge of the nature of glomerular function will be incomplete and the basis of our conceptions of the processes which are responsible for the formation of urine will contain assumptions which, however great their inherent probability, require the test of experiment before they can attain a wholly satisfactory degree of credibility. The discovery that the kidney of the living frog is accessible to direct and precise observation (1), the development of a satisfactory method for drawing fluid as it is separated from the blood within the capsule of Bowman, and the demonstration that this fluid can be collected without contamination and in measurable quantities (2) led to the hope that methods might be found reliably applicable to the quantitative study of this fluid. It is the purpose of this paper to describe experiments which may be regarded as the beginning of a realization of this hope.

Two questions concerning the glomerular fluid have been studied: (1) the molecular concentration as shown by determination of its freezing point; (2) chloride concentration. Our work on the former has completely failed of success; data have been secured concerning chlorides which we regard as worthy of record.

In exceptional instances, it has been possible to continue the

* Results contained in this paper were presented before the Eleventh International Physiological Congress at Edinburgh, July, 1923 (*Quart. J. Exp. Physiol., Suppl. Vol.*, 1923, 235).

collection of glomerular urine from a single capsule of Bowman for as long as 18 hours; in one instance the rate of collection of fluid was as high as 2.5 mg. per hour; but in the usual successful experiment the amount of fluid which can be taken from a glomerulus rarely amounts to more than 1 mg. per hour and one cannot as yet expect to prolong collections routinely for more than 4 or 5 hours. Experience showed that these figures were the exception rather than the rule, and hence we have aimed to develop methods which should be reliable when applied to as little as 1 to 2 mg. of fluid, or even less.

*Molecular Concentration.*—A brief description of our attempts to determine the $\Delta$ of small amounts of fluid is included for the sole reason that it may be of service to someone to whom the thought may occur, as it did to us, that this information might be relatively easy to obtain. A small piece of glass tubing, about 3 mm. inside diameter, was shaped in the flame so that one end was flattened and curved laterally so that it would fit the convexity of the stem of a Beckmann thermometer. A minute amount of N/10 NaCl was drawn into each of several glass capillary tubes about 5 cm. long, which had been scrupulously cleaned and dried. One end of each capillary was sealed with wax, the other inserted into the broad end of the glass tube mentioned above and sealed in place by plaster of Paris. The round end of the glass tube was connected by rubber tubing with a small rubber bulb. Intermittent slight pressure on the bulb caused the fluid in the capillary tubes to move up and down and we proposed to use the cessation or resumption of movement so induced as indicator of freezing or liquefaction. The glass tube with its attached capillaries was fastened to the stem of a Beckmann thermometer so that the column of fluid which each capillary contained was in closest possible proximity to the bulb. This was immersed in the usual fashion in a tube containing 20 per cent NaCl, which in turn was jacketed first with air and then with 20 per cent NaCl, the whole apparatus being arranged to stand in a freezing mixture. Mechanical stirring was employed for the inner and outer tubes, and the rates at which the mercury column fell (1° in 5 to 10 minutes) were extremely uniform and satisfactory. We soon found that we could not control the phenomenon of supercooling, which is more conspicuous in fluid contained in capillary tubes than in tubes of the size commonly used in freezing point estimations.

We then tried the plan of suddenly freezing the contents of the capillary tubes by immersion in carbon dioxide snow, followed by slowly raising the temperature, observing the temperature at which liquefaction occurred. The air jacket of the apparatus first used was replaced by a vacuum (Dewar) tube. The tubes immediately after freezing were transferred to the 20 per cent NaCl solution, previously cooled to below $-5$°C. Improved arrangements for control of temperature were made so that a uniformly

steady rise of 1° in 20 minutes was possible. Accurate and consistent results were obtained when distilled water was the fluid within the capillary tubes. But when N/10 or N/25 NaCl was used, six tubes being simultaneously tested in a single experiment, the results were not only far from the true freezing point of the solution, but were extraordinarily discordant. We therefore concluded that we were too ignorant of physical laws to make further work along this line productive.

*Chlorides.*—In the estimation of chlorides we were much more fortunate. The work of T. W. Richards and his colleagues (3), has furnished a method for estimation of Cl in extremely minute amounts with extraordinary accuracy. Since the method requires the most rigorous precautions against contamination or loss, and experience with the manipulations involved, we spent many weeks in working with known solutions until we were sure that we could control the various sources of error and could consistently obtain satisfactory results. All of the essential details of the method are to be found in the papers from the Harvard Laboratory, but since acceptance of our results will depend upon confidence in our ability to use the method it seems necessary to describe it in detail here.

Since the Cl concentration of normal frog's plasma is approximately 0.24 per cent, and since we hoped to obtain as much as 2 mg. of glomerular urine, the quantity of Cl to be estimated was assumed to be of the order of 0.005 mg. It is at once obvious that the common precautions observed in the current methods of macro or even micro analysis are inadequate for protection against error in dealing with such small amounts. T. W. Richards' method is nephelometric: and when in this description a substance or solution is described as Cl-free or pure, we mean that it has been tested in the nephelometer and found to be Cl-free within the limits of delicacy of our apparatus: and when a utensil is described as clean we mean that it has been adequately washed with pure water as shown by nephelometric tests. Richards could detect Cl in concentrations as low as 1 in 30,000,000: our instruments permitted its detection in concentrations lower than 1 in 10,000,000.

*Water.*—The distilled water used for making solutions and for cleansing apparatus was prepared by redistilling the usual laboratory supply in a room kept as free as possible from fumes and dust. The distilling flask, condenser, special adapter for preventing access of dust, and collecting bottle were all made of hard glass.

Every lot was scrupulously tested, not only at the time it was made but at frequent intervals during its use.

*Reagents.*—Sodium chloride was prepared by dissolving Merck's reagent NaCl in pure water, precipitating it by passing in gaseous HCl, filtering, and washing the crystals with pure water. The process was twice repeated, the final product dried to constant weight at 110°C., and kept in glass-stoppered bottles in the desiccator.

Silver nitrate (Merck's reagent) was recrystallized four times from pure water: solutions of the final product were Cl-free.

Baker's analyzed nitric acid was redistilled and the middle third of the distillate was found to be Cl-free.

Mercury, used for filling the apparatus with which glomerular urine was collected, was made Cl-free by repeatedly allowing it to flow in a fine stream through a column of pure water 5 feet long.

*Collection of Glomerular Urine.*—The technique of collecting glomerular urine was the same as that described in our previous paper (2). The surface of the frog's kidney was washed with distilled water before the drying preliminary to the insertion of the pipette into the capsule of Bowman. Quartz tubing, glass three-way tube, rubber tube, and levelling bulb were all scrupulously cleaned before assembling the collecting apparatus: this was then filled with Cl-free mercury in such a way that the mercury in the long arm and quartz tip of the pipette did not come in contact with the rubber tubing. In every instance, before insertion of the pipette, the quartz tip was again washed by drawing in and discharging pure water from it many times, and the final washing proved to be chloride-free. Careful estimation showed that the moisture which may remain adherent to the mercury and the inner wall of the quartz tip was insufficient to affect the results.

When collection of a sample of glomerular urine was finished, the surface of the kidney was again rinsed with pure water and dried before withdrawal of the pipette. The outside of the tip of the pipette was also rinsed with pure water before discharging the sample into a weighing tube.

The operation of collecting glomerular urine was performed in a room as remote from fumes as any in this building.

*Transfer and Weighing of Glomerular Urine.*—Capillary tubes, 15 to 20 cm. long and about 0.4 mm. inside diameter, were drawn

from clean resistance glass tubing. Each was provided with a cap, which consisted of another capillary about one-third the length of the first and of such inside diameter that the first could be inserted into it. One end of the cap was sealed in the flame. The capillary tube with its cap, both known to be Cl-free and both having been dried in the oven, were weighed. Then the tube without its cap was attached to the edge of a microscope slide by means of a small steel clamp held in position by plasticine, and this was placed on the stage of the binocular microscrope so that the upper open end of the tube was visible in the field. The tube was inclined so that it was more nearly horizontal than was the collecting pipette. Having brought both tip of pipette and upper end of capillary into the field of microscopic vision, the tip of the pipette was lowered by means of the Barbour stage so that it projected into the open end of the capillary without touching it. Then the three-way tap was suitably turned and the levelling bulb raised until the fluid in the pipette was discharged into the capillary. It was easily possible to make the transfer without wetting the inner wall of the glass capillary within $\frac{1}{8}$ inch of its end.

The pipette was then withdrawn, the fluid in the capillary tube allowed to flow down into it for an inch or so, the lower end sealed in the flame, and the upper end inserted into the cap. The tube, cap, and contents were then weighed. A Troemner balance was used, advertised to be sensitive to 0.05 mg., but certainly sensitive to 0.02 mg. as we used it. Control tests were made which showed that a minute amount of fluid kept at room temperature in such a tube with cap did not lose weight by evaporation in several hours. Our weighings of glomerular urine were always made immediately after transfer.

*Estimation of Cl.*—All the details of analysis were conducted in a small room, 5 feet square and 8 feet high, having one door and no windows. The walls and ceiling were covered with black cloth. An hour or two before beginning an analysis the cloth and the floor were thoroughly sprayed with water. While making the analyses, each of us wore two laboratory gowns, the outer one of which was soaking wet with water. It was only after the adoption of these precautions that we were consistently able to avoid contamination of our solutions with dust.

T. W. Richards' method for Cl consists of nephelometric comparison of the opalescence or turbidity produced by adding pure silver nitrate to the unknown solution with that similarly produced in a standard solution of NaCl. The optimal conditions for production of turbidity and for accuracy of comparisons are described in Richards' papers and in that of Wells, and were those which we adopted. All solutions used were kept in glass-stoppered, glass-capped, hard glass bottles in the dark, dust-free room mentioned above. The measuring implements and nephelometer were also kept there: and the final washing of these implements, the preparation of turbidities, and the nephelometric readings were made there under the conditions already mentioned.

*Solutions.*—A stock solution of pure NaCl was made by dissolving 1.25 gm. of the purified NaCl in 500 cc. of pure water. This was diluted 2000 times to make the standard used in the actual estimations (1 cc. = 0.00125 mg.) and this dilution was delivered in appropriate amounts into the volumetric flasks from a 25 cc. certified burette which could be read with an accuracy of 0.02 cc.

The Cl-free silver nitrate was dissolved in pure water to make a 0.002 N solution. It was delivered into the volumetric flasks from a 50 cc. burette which could be read with an accuracy of 0.05 cc.

0.1 N nitric acid was prepared from the Cl-free supply, diluted with pure water. It was delivered into the volumetric flasks from a 25 cc. burette.

The nephelometer (Kober) used was made by the Klett Manufacturing Company of New York and in the many blank tests and control estimations was found satisfactory. The cups, especially made for this work, had a capacity of 4 cc. on the narrow part.

When blank tests were made, the cups being filled with pure water and the prisms set at 40 mm., no light was visible through the eyepiece for several minutes after entering the dark room: after that, the faintest gray circle became apparent. Identical results were obtained when any of the reagents described were similarly tested either alone or in mixtures of different proportions (with the obvious exception of mixtures of the NaCl and $AgNO_3$).

*Conduct of Analysis.*—The sample of glomerular urine, after its weight had been determined as above described, was transferred to a clean certified 25 cc. volumetric flask as follows:

The end of the capillary tube which had not been protected by the cap was thoroughly rinsed with pure water. The sealed tip of that end was broken off with clean scissors. That end of the capillary was then held in contact with the inner surface of the neck of the volumetric flask and the other was brought in contact with drops of pure water as they slowly issued from the tip of the glass outlet tube of the pure water supply bottle kept in the dark room. Capillarity caused the entire stream to pass rapidly and cleanly through the tube into the flask, and in this way the contents of the tube were quantitatively transferred to the flask and the tube itself quantitatively washed. In a successful transfer no water passed over the outside of the tube into the flask. More water was then added to the flask to make the volume of its contents about 10 cc. It was stoppered and set aside until the flasks of standard NaCl could be prepared.

A volume of standard NaCl solution, judged to be sufficient to contain an amount of NaCl of the same order as that in the glomerular sample, was then measured into a second 25 cc. volumetric flask. To guard against an error of judgment a number of flasks were prepared, sometimes as many as six, containing different amounts of NaCl. The contents of these flasks were brought to approximately the same volume—not more than 15 cc. 2 cc. of N/10 nitric acid was then added to each of these and to the flask containing the glomerular urine; each was shaken carefully and allowed to stand for 5 minutes. Then to each flask in turn was added 5 cc. of 0.002 N silver nitrate. It is essential that the contents of the different flasks be agitated in the same manner during this process. Since time is a factor in the development of maximal opalescence the addition of the silver nitrate was made as expeditiously as was prudent, the flask containing the glomerular urine being placed in the middle of the series. Water was then added to each flask to make exactly 25 cc. and the contents of each thoroughly mixed by inverting twenty-five times.

By gross observation of the opalescence produced in the different flasks, it was usually possible to choose the proper flask of standard NaCl with which to compare the unknown. The nephelometer cups and prisms, previously thoroughly cleaned and dry on the outside, were then given a final series of washings with pure water, rinsed three times each with the mixture which it was to receive, and the

TABLE I.

*Estimations of the Cl Content of Small Amounts of a Known Solution of Pure NaCl (1 Cc. = 2.5 Mg.).*

| Date. | Weight of solution taken. | NaCl content. | NaCl found. | Error | Nephelometer readings. | | Standards. |
|---|---|---|---|---|---|---|---|
| | | | | | Standard. | Unknown. | |
| *1923* | *mg.* | *mg.* | *mg.* | *per cent* | | | |
| Apr. 2 | 4.35 | 0.010875 | 0.01088 | +0.046 | 30 | (W) 35.0,34.3,34.7,33.9,35.1,34.4, 34.0,34.7,34.3,33.9,34.4,34.9: Average 34.47. | Standard turbidity made with 10 cc. standard NaCl = 0.0125 mg. |
| | | | 0.010765 | −1.0 | 31 | (R) 35.9,34.3,34.4,35.9,34.5,34.7, 35.1,34.9: Average 34.96. | |
| " 2 | 2.75 | 0.006875 | 0.00704 | +2.2 | 30 | (W) 26.0,26.8,26.5,26.9,26.6,26.8: Average 26.6. | Two standard turbidities made (1) 10 cc. NaCl = 0.0125 mg. (2) 5 cc. NaCl = 0.00625 mg. Standard (2) used. |
| | | | 0.00662 | −3.7 | 30 | (R) 28.5,27.7,28.7,27.7,28.5,27.5, 28.2,28.9,29.0: Average 28.3. | |
| " 3 | 2.6 | 0.0065 | 0.00665 | +2.3 | 30 | (W) 27.9,27.8,27.5,28.8,28.0,28.3, 27.8,28.8,28.1,28.9: Average 28.19. | Three standard turbidities made: (1) 7.5 cc. NaCl = 0.009375 mg. NaCl. (2) 5.0 cc. NaCl = 0.00625 mg. NaCl. (3) 2.5 cc. NaCl = 0.003125 mg. NaCl. Standard (2) used. |
| | | | 0.00682 | +4.9 | 30 | (R) 28.0,27.0,27.7,28.0,27.0,27.8, 27.3,28.2,27.2,26.7: Average 27.49. | |
| " 3 | 4.6 | 0.0115 | 0.01176 | +2.3 | 30 | (W) 28.5,29.0,28.6,29.3,27.6,28.7, 29.7,28.4,28.0,29.2: Average 28.7. | Two standard turbidities made: (1) 9 cc. NaCl = 0.01125 mg. NaCl. (2) 10 cc. NaCl = 0.0125 mg. NaCl. Standard (1) used. |
| | | | 0.01221 | +6.2 | 30 | (R) 29.3,26.7,27.9,27.3,27.0,28.1, 27.0,28.0,27.9, 27.2: Average 27.64. | |
| " 7 | 3.5 | 0.00875 | 0.008813 | +0.7 | 30 | (W) 31.0,30.5,29.6,29.0,29.0,30.0, 29.6,29.8,29.1, 29.0: Average 29.66. | Standard turbidity = 7 cc. NaCl = 0.00875 mg. NaCl. |
| | | | 0.008846 | +1.4 | 30 | (R) 29.7,29.8,29.7,28.6,28.6,30.2, 28.3,29.5: Average 29.3. | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | 28.3,29.5: Average 29.3. | |
| Apr. 7 | 5.96 | 0.0149 | 0.0152 | +2.0 | 30 | (W) | 30.0,29.5,29.0,30.2,29.9,29.0, 30.3,29.8,29.0,30.1: Average 29.68. | Standard turbidity = 12 cc. NaCl = 0.0150 mg. NaCl. |
| | | | 0.01483 | −0.5 | | (R) | 31.0,30.5,30.0,30.0,29.9,30.4, 30.9,30.0,30.6,30.0: Average 30.33. | |

Wearn's greatest error, 2.3 per cent: average error, 1.6 per cent.
Richards' " " 6.2 " " " " 3.0 " "
Average error of all, 2.3 per cent.

cups finally filled with the mixture. In the final washing and in the filling of the nephelometer cups, it is highly important that no fluid collect on the outer surface and equally important that the fingers shall not come in contact with any portion of the glass surface through which light is subsequently to enter. The prism which was inserted into the tube containing the standard was usually set at 30 mm. From three to twelve readings were usually made by each of us, the final result being the average of all.

*Control Tests of Method.*—After each detail of the method had been studied separately, a series of control estimations of the Cl content of a known NaCl solution was made, in each of which was included every manipulation required in the actual analysis of glomerular urine, with the single exception of collection of sample from a capsule of Bowman. The known NaCl solution (1 cc. = 2.5 mg. NaCl) was drawn from a small open cylinder into the capillary pipette in amount comparable to that of the glomerular urine samples which we hoped to collect. It was discharged into a weighed capillary weighing tube, weighed, transferred, and analyzed exactly as has been described. In Table I are contained the results and essential data of the last six consecutive tests made before beginning work with the glomerular urine. They show, for Wearn, maximum error of 2.3 per cent; average error, 1.6 per cent: for Richards, maximum error of 6.2 per cent; average error, 3.0 per cent: average error of all estimations, 2.3 per cent.

On the basis of these results we concluded that we had become sufficiently proficient with the method to justify us in proceeding with the analysis of glomerular urines.

*Analysis of Bladder Urine and of Blood Plasma.*—Whitehorn's method (4) was used without modification. M/35.46 solutions of $AgNO_3$ and KCNS were made for us by Dr. J. B. Brown and were standardized and adjusted by us against solutions of the pure NaCl used in the micro determinations described above. Urine and plasma were measured in a 1 cc. pipette, graduated in 1/10's, which had been accurately calibrated both for water and for frog's plasma. Plasma proteins were precipitated according to Folin and Wu (5). Since the amounts of plasma available were small we were obliged to adopt centrifugation of the warmed mixture rather than filtration in order to obtain sufficient clear

protein-free fluid. This was conducted in tightly stoppered tubes to prevent evaporation.

*Collection of Blood Plasma.*—In eight of the thirteen experiments described and tabulated, blood plasma was obtained by centrifuging all of the blood which could be taken from the aorta of the frog at the end of an experiment. No especial precautions were employed to prevent loss of $CO_2$ during centrifugation. It is obvious that there could be no complete certainty that the Cl content of blood collected all at once at the close of an experiment was identical with the true average Cl content of the blood during its circulation through the glomerular capillaries during the hours of collection of glomerular urine. To meet these two objections, the following procedure was developed and applied in three experiments (Nos. 11, 12, and 13).

After preparing the frog for puncture of a capsule of Bowman, one arch of the aorta was carefully dissected, tied, clamped, and a paraffined cannula inserted. The cannula was about 4 inches long and was bent to form a right angle: the end which was not attached to the aorta was thrust through one hole of a two-hole rubber stopper and this in turn was inserted into the end of the blood collection tube. This was simply a small test-tube cut to appropriate length, capacity, 4 cc. One arm of another bent glass tube passed through the other hole of the rubber stopper, the other arm of this tube being connected with a 2 cc. pipette, graduated in 1/10's, by means of small bore rubber tubing. The pipette, from its lowest mark to the tip, the rubber tubing and glass tube, the collecting tube, and cannula were completely filled with liquid paraffin before inserting the cannula into the aorta. After this had been done, the collecting tube was firmly strapped to the edge of the frog board with adhesive tape, liberally smeared with lead plaster. Then a clamp was put on the rubber tube between the pipette and the collecting tube and the clamp on the aorta removed. This arrangement permitted us to take blood in any desired quantity from the arterial system at intervals during the period of collection of glomerular urine without disturbing arrangements for the latter in the slightest degree. The blood was protected from contact with air by the paraffin oil and at the end of the collection was centrifuged before removal of the layer of oil above it. A few grains of heparin placed in the bottom of the collecting tube were effective in preventing clotting.

Bladder urine was collected at the end of an experiment by holding up the wall of the bladder with forceps, thrusting the sharpened point of a pipette through the wall, and removing the urine by suction.

*Other Experimental Details.—Rana pipiens* and *Rana catesbiana* were the species of frogs used. In all except two experiments anesthesia with urethane was substituted for pithing. 1 cc. of 20 per cent solution injected into the dorsal lymph sac produced anesthesia in a frog of 40 to 60 gm., but was insufficient to suppress muscular movements completely during a long experiment. Hence, at the beginning of each experiment a small, pointed glass tube, connected with a syringe by about a foot of small rubber tubing, was thrust through the skin of one thigh and left in place during the whole experiment. By this means additional urethane could be injected during the course of the experiment without touching the animal. The same means were employed to introduce glucose or salt solution.

In order to prevent progressive failure of the frog's circulation during an experiment it was necessary to guard against loss of water by evaporation. The skin was covered with wet cloths and the frog holder arranged so that the frog lay in a shallow pool of water. When subcutaneous injection of fluid was necessary, 0.5 or 1.0 per cent glucose was used, except in those experiments in which the effect of NaCl was tested.

Not infrequently frogs were encountered in which the venous system was dilated, the glomerular circulation sluggish, and collection of glomerular fluid impossible: sometimes, despite fairly active glomerular circulation, little glomerular fluid accumulated. In these cases only transient improvement followed the introduction of water or solutions of glucose. In some, but by no means all of these animals, marked improvement was effected by applying a few drops of pituitrin solution (1/500,000 to 1/100,000) to the surface of the kidney or by introducing it into the abdominal cavity. The effect consisted in conspicuous increase in rate and volume of blood flow through the glomerular capillaries, apparently the result of dilatation of the smaller renal arterioles; at the same time venous congestion was diminished. This device prevented failure in a number of experiments made in the months of May, June, and July; it was completely ineffective in a series of wholly unsuccessful experiments made in the month of October.

The results of thirteen experiments in which glomerular urine has been analyzed are grouped in Table II, together with data concerning the preparation of the frogs. The analytical details of these experiments are included in the data on pp. 270 to 273. All results are expressed as sodium chloride.

Two corrections have been introduced into the figures for plasma chloride in Table II in order to make them more accurately comparable with those for glomerular urine. The plasma was measured volumetrically, whereas glomerular urine was weighed. We have, therefore, introduced a correction of 1 per cent, based on the fact that in one estimation the specific gravity of frog's plasma was found to be 1.013. In order to take account of the fact that plasma, because of its protein content, contains approximately 2.5 per cent less water than does glomerular urine, we have divided the weight percentages found after the first correction by 0.975. The sum of these two corrections is equal to only 1.5 per cent and is negligible in so far as our conclusions are concerned.

*Miscellaneous Observations.*

Certain other results and observations than those contained in the table are of interest. From the figures for rate of glomerular fluid collection, some idea of the rate of glomerular elimination of fluid can be obtained. In four experiments in which it was most rapid, the average rate of collection was 0.9 to 1.0 mg. per hour. The rate of glomerular elimination was certainly higher than this because the tubule was not obstructed. In one experiment, however, by a fortunate accident, the opening from the capsule into the tubule was obstructed by a minute globule of mercury which was forced out of the tip of the pipette into the capsule when the pipette was introduced. That the exit of glomerular fluid into the tubule was actually obstructed was obvious from the distention of the capsule which began immediately. In approximately half an hour 0.58 mg. of glomerular urine was collected from this capsule. From these figures we are certainly justified in saying that glomerular fluid may be formed at rates exceeding 1 mg. per hour per glomerulus.[1]

[1] This observation furnishes direct contradiction of the contention of Woodland that the glomerulus is not the site of elimination of fluid from the blood (Woodland, W. N. F., *J. Asiatic Soc. Bengal*, 1922, N. S., xviii, 121; Am. J. Physiol., 1922–23, lxiii, 368).

TABLE II.

*Chloride Content of Frog's Plasma, Glomerular Urine, and Bladder Urine.*

| Experiment No. | Date. | Weight of frog. | Preparation of frog. | Time of collection and weight of glomerular urine. | NaCl content of. | | | Criticisms and remarks. |
|---|---|---|---|---|---|---|---|---|
| | | | | | Plasma. | Glomerular urine. | Bladder urine. | |
| | | *gm.* | | | *per cent* | *per cent* | *per cent* | |
| 1 | Apr. 12 | | In distilled water 24 to 48 hrs. before experiment. Pithed. | 0.51 mg. | | 0.25 | | Glomerular urine dried in tube. Transfer may not have been complete. |
| 2 | " 16 | 50 | 1 cc. 5 per cent $NaHCO_3$ + 2 cc. $H_2O$. Pithed. | 5 hrs. 2.25 mg. | | 0.34 | 0 | Standard not near enough like unknown. |
| 3 | May 9 | 52 | Urethane. Pituitrin. 2 cc. 1 per cent glucose at beginning of experiment. | 5½ hrs. 0.53 mg. | 0.36 | 0.46 | 0.025 | May have been slight evaporation during transfer of glomerular urine. |
| 4 | " 10 | 42 | Urethane. Pituitrin. 2 cc. 1 per cent glucose. | 3½ hrs. 1.59 mg. | 0.39 | 0.45 | 0 | Probably slight evaporation during transfer of glomerular urine. |
| 5 | " 11 | 43* | Urethane. Pituitrin. 2 cc. 1 per cent glucose. | 4¼ hrs. 1.38 mg. | 0.33 | 0.43 | 0.008 | |
| 6 | " 14 | 46* | Urethane. Pituitrin. | 3¾ hrs. 1.07+ mg. | 0.30 | 0.33 | | |
| 7 | " 16 | 87 | Urethane. Pituitrin. 2 cc. 1 per cent glucose. 1 cc. $H_2O$. | 1¾ + 3¾ hrs. 1.12 mg. | | 0.38 | 0.08 | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 8 | May 17 | 67 | Urethane. Pituitrin. 5 per cent NaCl as follows: 1 cc. ½ hr. before and 1 cc. ½, ¾, and 1¼ hrs. after beginning collection. | 5 hrs.<br>1.04 mg. | 1.21 | 1.21 | 1.02 | Bladder urine taken before beginning of experiment contained no Cl. |
| 9 | " 18 | 67 | Urethane. Pituitrin. 2 per cent NaCl. 1 cc. 15 min. before collection, 0.5 cc. 25 min., 55 min., and 1 hr. 55 min. after collection began. | 2⅔ hrs.<br>2.84 mg. | 0.48 | 0.98 | 0.26 | Glomerular urine made up in 50 cc. flask: quantities of $HNO_3$ and $AgNO_3$ doubled. Bladder urine at beginning of experiment contained 0.04 per cent NaCl. |
| 10 | " 21 | 47 | Urethane. Pituitrin. 1 cc. water by stomach. 5 per cent NaCl, 1 cc. 50 min. before and 0.5 cc. 10 min. before collection. 0.5 cc. 20 min. after and 0.2 cc. 40 min. after collection began. | 1 hr. 35 min.<br>1.36 mg. | 0.68 | 0.85 | 0.27 | |
| 11 | " 31 | 65 | Urethane. 1 per cent NaCl 1 cc. 1½ hrs. before beginning, 1 cc. at beginning, and 0.5 cc. 1 hr. after beginning collection. | 5.15 to 7.35 = 2⅓ hrs.<br>2.01 mg. | 0.47<br>(0.48)† | 0.54 | | Blood collected as follows: 5.20, 0.2 cc.; 5.50, 0.2 cc.; 6.05, 0.2 cc.; 6.23, 0.25 cc.; 6.45, 0.2 cc.; 7.05, 0.23 cc.; 7.26, 0.17 cc. |

TABLE II—*Concluded.*

| Experiment No. | Date | Weight of frog. | Preparation of frog. | Time of collection and weight of glomerular urine. | NaCl content of. | | | Criticisms and remarks. |
|---|---|---|---|---|---|---|---|---|
| | | | | | Plasma | Glomerular urine. | Bladder urine. | |
| | | *gm.* | | | *per cent* | *per cent* | *per cent* | |
| 12 | June 1 | 65 | Urethane. Pituitrin. 1 per cent glucose, 1 cc. at 11.00, 1 cc. at 11.25, 0.5 cc. at 12.10. | 11.45 to 3.40 = 3 hrs. 55 min. 2.45 mg. | 0.31 (0.30)† | 0.33 | 0 | Blood collected as follows: 11.55, 0.1 cc.; 12.25, 0.13 cc.; 12.55, 0.2 cc.; 1.20, 0.2 cc.; 1.50, 0.25 cc.; 2.25, 0.2 cc.; 2.53, 0.23 cc.; 3.20, 0.2 cc. |
| 13 | " 2 | 63 | Urethane. 1 per cent glucose, 2 cc. before beginning collection. | 12.30 to 2.20 = 1 hr. 50 min. 1.19+ mg. | 0.31 (0.33) | 0.43 | | Blood collected as follows: 12.32, 0.15 cc.; 12.52, 0.2 cc.; 1.20, 0.25 cc.; 1.40, 0.25 cc.; 2.10, 0.25 cc. Small amount of Hg transferred with glomerular urine to capillary weighing tube: hence second transfer to new weighing tube necessary. Possibility of slight loss by evaporation. |

* Weight of frog after removal of ovaries, kidneys, and blood.
† Chloride in plasma of blood taken at the end of the experiment.

Our data are insufficient to permit a useful discussion of the relation of the rates of glomerular urine collection and accumulation of urine in the bladder. In some experiments, however, a high rate of glomerular collection was found to coincide with a low rate of elimination from the kidney.

The total number of glomeruli in one kidney from each of the frogs used in the experiments of May 14 and 16 were counted for us by Dr. Hayman and Dr. Starr by Nelson's method (6). The numbers were 3650 and 5460, respectively.

During the course of these experiments many observations have been made concerning variations in the number of glomeruli through which blood flows simultaneously, concerning intermittence of glomerular flow, and concerning different types of blood flow in different glomeruli. The experience of Richards and Schmidt (1) has been confirmed.

In the four experiments in which NaCl was injected the glomerulus from which fluid was taken exhibited slow, pulsating blood flow. In our past experience, this type of blood flow has been associated with scanty collection of urine and frequent clogging of the pipette. In these instances glomerular urine collections were abundant. This experience may be connected with the observation that the bulk of the tungstic acid precipitates of proteins from the plasma of these frogs was extremely scanty, indicating an unusual degree of dilution of the plasma. The observations indicate the extent to which blood flow, as an element in urine formation, may be outweighed by other factors.

## DISCUSSION.

The results presented show conspicuous difference between the chloride content of bladder urine and that of glomerular urine. In three experiments (2, 4, and 12), in which no chloride was found in bladder urine, glomerular urine contained from 0.33 to 0.45 per cent: in three others (3, 5, and 7), chloride in bladder urine ranged from 0.008 to 0.08 per cent, while glomerular urine contained from 0.32 to 0.46 per cent. These differences are much greater than the probable errors of the methods used and furnish decisive confirmation of our earlier statements based upon qualitative tests (2). The facts of glomerular elimination and tubular

reabsorption of chloride in the frog's kidney would appear to be definitely established.

In Experiments 8, 9, and 10, sodium chloride in various amounts was injected subcutaneously before and during collection of glomerular and bladder urines. In each instance, the bladder urine chloride was conspicuously less than glomerular urine chloride. This observation is in harmony with previous results (7) which have shown the inability of the frog's kidney to concentrate chloride. It shows that chloride must have been reabsorbed from the urinary tubule at a faster rate than water, and it indicates that other factors than the concentration of chloride in the plasma take part in the control of chloride reabsorption.

The chief interest in these experiments is to be found in the opportunity, afforded for the first time, of comparing the chloride concentration of the blood plasma with that of the glomerular fluid. In the majority, it is seen that the results of such comparison are at variance with the chief assumption contained in the filtration theory of glomerular function as commonly accepted. In every experiment save one the figure for glomerular Cl is higher than for plasma Cl. In only three (6, 8, and 12) is this difference within 10 per cent of the plasma chloride—a difference which we concede may be within the range of experimental error. In six (3, 4, 5, 10, 11, and 13) the difference amounts to from 15 to 39 per cent of the plasma chloride: in one (9), glomerular Cl is more than double the plasma Cl.

Before these figures can be accepted as truly representing the facts it is necessary to scrutinize carefully every possible source of error. Suspicion will be directed first at the figures for glomerular chloride because the amounts of Cl estimated are much smaller than those with which physiological investigations are usually concerned. Both the theoretical and practical soundness of the nephelometric method of estimating minute amounts of Cl have been amply demonstrated by T. W. Richards and his colleagues. Our capacity to use it competently may very naturally be called into question. It is for this reason that we have described all of our manipulations in as complete detail as possible. This description and the results of control analyses presented in Table I are all that we can offer in this connection except the statement that as our experience with the method increased so also

did our feeling of confidence and certainty with it increase—a feeling which came to be consistently borne out by the results.

The Whitehorn method for plasma chloride has been subjected to careful study, not only by its author, but by others since. The work of Greenwald and Gross (8) indicated its superiority to other commonly used methods. Since our work was done, Van Slyke (9) has developed an open Carius method for plasma chloride in which the disadvantages attendant upon precipitation of the plasma proteins are avoided. His figures contain evidence of the reliability of the silver titration under the conditions specified by Whitehorn. These two methods have been subjected to the most careful comparison in this laboratory; Dr. Anna Muschel worked with known NaCl solutions and dog's plasma; Dr. Benjamin Freeman with known chloride solutions and with dialyzed horse serum to which known amounts of NaCl were added. Their results show uniform agreement within less than 1 per cent.

The solutions of $AgNO_3$ and KCNS used by us in the Whitehorn method were standardized against the solution of pure sodium chloride (1 cc. = 2.5 mg.) from which the dilutions used as standards in the nephelometric method were prepared. At frequent intervals during the work they were tested against each other and against known chloride solutions with results which were always within 1 per cent of theory. We are unable to discover in our use of the Whitehorn method a source of error so gross as to account for the differences which concern us.

In our previous paper it was shown that the glomerular fluid, collected under conditions identical with those used in these experiments, contained no protein detectable by acetic acid and potassium ferrocyanide. Confirmation of this statement has been repeatedly made by us since. Occasionally, however, it was found that the tip of the collecting pipette became clogged, presumably with coagulated protein. This happened in instances in which glomerular blood flow became excessively slow. No such occasions are included in the experiments here under discussion. It is understood, of course, that a trace of protein in the fluid subjected to nephelometric Cl estimation might yield opalescence when treated with $HNO_3$ and $AgNO_3$. A single experiment was made in which 0.04 cc. of N/10 NaCl in one flask and 0.04 cc. of undiluted frog's plasma in another were diluted

with pure water and treated with $HNO_3$ and $AgNO_3$, all in the same proportions as in the nephelometric estimations. The nephelometric reading of the NaCl mixture was 40; that of the plasma mixture, 31. This means that all other substances than Cl in the undiluted plasma produced turbidity equal to about one-half that produced by the chloride. The effect, therefore, of traces of protein in the glomerular fluid, were they present, would be negligible.

While we are unable to point out possible sources of error in our analytical methods sufficient to account for the observed differences in chloride contents of plasma and glomerular urine, it is not so certain that errors are not inherent in our plan of experiment. In the first seven of the ten experiments in which both plasma Cl and glomerular urine Cl were estimated, the plasma was taken from blood withdrawn at the end of the collection of glomerular urine. Possibly the plasma so obtained was not identical in composition with that from which the glomerular urine was derived. Three experiments were made to test this.

Three large frogs (60 to 80 gm.) were anesthetized with urethane. One arch of the aorta of each was exposed and 1 cc. of blood drawn and set aside for separation and analysis of plasma. Two of the frogs were then subjected to the operation which is required in the exposure of the kidney for capsular puncture. The third was not. Each was injected subcutaneously with 2 cc. of 1 per cent glucose solution, and the skin kept covered with cloths wet with water. Approximately 4 hours later blood was again withdrawn, the plasma separated and analyzed.

| | First bleeding. | | Second bleeding. | |
|---|---|---|---|---|
| | Cells. | Plasma NaCl. | Cells. | Plasma NaCl. |
| | *per cent* | *per cent* | *per cent* | *per cent* |
| Frog 1..... | 28.1 | 0.39 | 11.1 | 0.33 |
| Frog 2..... | 38.0 | 0.39 | 13.2 | 0.31 |
| Frog 3..... | 29.0 | 0.44 | 16.3 | 0.34 |

In another experiment a large frog was anesthetized with urethane and 1.2 cc. of blood taken from the aorta (Sample 1), 2 cc. of 1 per cent glucose solution was then injected subcutaneously and the skin covered with wet cloths. 2 hours later 1.25 cc. of blood

was taken (Sample 2). Again 2 cc. of glucose was injected and after another 2 hour interval blood was drawn (Sample 3). The cell contents and plasma chloride percentages of the three samples were as follows:

| | *Sample 1.* | *Sample 2.* | *Sample 3.* |
|---|---|---|---|
| Cells (*per cent*) | 36.7 | 35.2 | 15.6 |
| Plasma chloride (*per cent*) | 0.45 | 0.43 | 0.31 |

These experiments indicate that during the course of a long experiment in which glomerular urine is collected and in which absorption of Cl-free fluid is freely permitted, progressive changes in the composition of the blood may develop to such an extent as to vitiate conclusions drawn from the composition of blood taken at the end of the experiment.

The facts and considerations just outlined led to the adoption of the method for collecting blood described on page 257. It enabled the removal of a little blood at frequent intervals during the glomerular urine collection. Three experiments (11–13) were made in which this method was used. In only one (12) did the glomerular urine chloride exceed plasma chloride by less than 10 per cent: in Experiment 11 this excess was 15 per cent and in Experiment 13 it was 39 per cent.

Moreover, in addition to the composite blood sample taken as described, a large sample of blood was drawn at the end of each of these experiments. In each case the Cl content of this was practically identical with that of the composite. This indicates that the decrease in plasma chloride which was demonstrated in the supplementary experiments just cited may not have occurred in the majority of experiments in our main series.

Question has often been raised with us concerning the possibility of contamination of the collected glomerular fluid with contents of the tubule into which the punctured capsule opens. We have given reasons for believing that this is not the case. Such evidence as we have justifies the belief that the chloride concentration of fluid in the tubules is less than that in the glomerular fluid. If our glomerular collections had been drawn partly from the tubule we might reasonably expect to have encountered lower chloride percentages.

Apart from considerations of possible technical errors, one reason at least may be advanced for thinking that the glomerular fluid

should show higher Cl concentration than the plasma from which it is derived.

It seems obvious that the Donnan phenomenon must play a part in determining the salt concentration of a protein-free fluid which is derived from a fluid containing both proteins and salt by passage through a membrane impermeable to the former but not the latter. We have no data concerning the alkali-binding power of frog's plasma proteins: but if we may use the figures of Van Slyke, Wu, and McLean (10) for the binding power of horse serum protein, together with White's figures (11) for pH and protein concentration of frog's plasma, an approximation of differences of salt concentration may be obtained. A solution containing 2.8[2] per cent horse serum protein at pH 7.65[2] (sodium being the alkali with which it is combined) and 0.4 per cent NaCl should, if equilibrium according to the Donnan formula exists, yield a protein-free filtrate containing 0.416 per cent NaCl.

We do not assume that this approximation derived from simplified conditions can adequately represent the degree to which the implications of Donnan's law may be applicable to the process of glomerular filtration. It is cited simply to show the extent to which the proteins of plasma may influence the composition of a glomerular filtrate. It seems proper to think that the Donnan phenomenon, in such an intricate system as blood, circulating through capillaries, should include not only substances to which the membrane is impermeable, but also substances to which the membrane shows varying degrees of permeability. Hence, it is impossible at present to make a satisfactorily complete application of the Donnan principle to the glomerular process.

The result given above, which was obtained by substituting in Donnan's formula values derived from the data of Van Slyke, Wu, and McLean and White, explains an excess of chloride in glomerular filtrate over that in plasma due to the presence of plasma proteins of the order of 4 per cent—an excess far less than that actually encountered in the majority of our experiments.

The outcome of this discussion is this:—We are confronted with data concerning the Cl contents of plasma and glomerular urine which do not agree with a well established conception of glomerular function. We are unable to discover flaws in analytical tech-

[2] White's figures.

nique sufficient to explain observed differences. In seven out of ten experiments the blood was not collected at the same time as was the glomerular fluid. A group of special experiments showed the possibility of error from this cause: but in two of the experiments in which this source of error was in part, at least, obviated similar discrepancy appeared: and in addition it was found that the Cl concentration of the final blood sample was practically identical with that of the composite sample obtained during the collection of glomerular fluid. In an ideal experiment, blood should be collected continuously at rates proportionate to the rates of elimination of glomerular urine. This ideal cannot be realized, but the evidence which we have indicates no gross distortion of the facts as a result of our deviations from it.

The Donnan formula, in so far as we are able to apply it, is inadequate to solve the difficulty.

The results may serve as the basis of reasonable doubt of the complete adequacy of the present conception of glomerular filtration: they indicate the fashion in which more perfect experiments may be made. Until such experiments have been made and have confirmed our results, we feel that a theoretical discussion of factors involved would be premature. Preliminary work for further investigations of this question is in process in this laboratory.

SUMMARY.

1. The nephelometric method of T. W. Richards for the estimation of minute amounts of Cl has been applied to the study of the chloride concentration of the glomerular urine, collected from frogs in thirteen experiments under various conditions.

2. Comparison of the Cl concentrations of glomerular and bladder urines simultaneously eliminated confirms the qualitative evidence previously advanced by us in proof of glomerular elimination and tubular reabsorption of Cl. The figures show that Cl is absorbed at a faster rate than water, even when plasma chloride is more than double its normal value as a result of injection of NaCl.

3. In ten of thirteen experiments the plasma Cl concentration was also determined. In all but one of these, glomerular Cl was higher than plasma chloride. In seven, the difference amounted to more than 15 per cent of the latter; in four, more than 28 per cent.

4. Conceding the possibility of errors of technique amounting

to 10 per cent, we are forced to conclude that the filtration theory, as at present defined, is not adequate to explain our results. If subsequent work shall confirm these, it is clear that the processes concerned in glomerular filtration require further study.

*Analytical Details.*

*Experiment 1.*—April 12. Weight of glomerular urine = 0.51 mg. Three standards prepared: (1) 1 cc.; (2) 1.5 cc.; (3) 2 cc. NaCl. No. 1 chosen = 0.00125 mg. NaCl. Prism in standard set at 30 mm.

*Readings:* (W) 29.0, 27.0, 28.2, 26.9, 28.0, 28.6, 29.0, 28.9.
Average 28.2 = 0.26 per cent.
(R) 32.1, 32.8, 29.0, 31.3, 30.9, 32.6.
Average 31.45 = 0.23 per cent.

*Plasma* and *bladder urine* not obtained.

*Experiment 2.*—April 16. Weight of glomerular urine = 2.25 mg. Four standards: (1) 15 cc.; (2) 12 cc.; (3) 9 cc.; (4) 5 cc. NaCl. No. 3 chosen = 0.01125 mg. NaCl. Prism in standard set at 25.

*Readings:* (W) 36.3, 37.0, 37.3, 37.1, 36.9, 36.5, 37.6, 37.2.
Average 36.99 = 0.34 per cent.
(R) 37.5, 36.5, 36.7, 37.5, 36.5, 36.6, 37.0, 36.5.
Average 36.85 = 0.34 per cent.

*Bladder urine*, 0.48 cc.: $AgNO_3$, 3.0 cc.: KCNS, 3.0 cc.: Cl = 0.

*Experiment 3.*—May 9. Weight of glomerular urine = 0.53 mg. Three standards: (1) 1.0 cc.; (2) 1.5 cc.; (3) 2.0 cc. NaCl. No. 2 chosen = 0.001875 mg. NaCl. Prism in standard set at 30 mm.

*Readings:* (W) 23.6, 22.8, 22.5, 23.0, 24.0, 23.4, 23.8, 24.3.
Average 23.425 = 0.45 per cent.
(R) 24.0, 21.6, 22.2, 22.3, 22.6, 22.8.
Average 22.57 = 0.47 per cent.

*Plasma*, 0.65 cc.: 4.5 cc. supernatant fluid titrated: $AgNO_3$, 3.0 cc.: KCNS, 2.05 cc.: NaCl = 0.35 per cent.

*Bladder urine*, 0.65 cc.: $AgNO_3$, 3.0 cc.: KCNS, 2.9 cc.: NaCl = 0.025 per cent.

*Experiment 4.*—May 10. Weight of glomerular urine = 1.59 mg. Probable slight evaporation during transfer. Four standards: (1) 4 cc.; (2) 5 cc.; (3) 6 cc.; (4) 7 cc. NaCl. No. 3 chosen. Set at 30.

*Readings:* (W) 32.0, 31.1, 31.7, 32.4, 32.6, 31.6, 32.0, 32.0.
Average 31.9 = 0.45 per cent.
(R) 31.2, 31.1, 31.1, 31.7, 31.7, 31.3, 32.1, 31.7.
Average 31.49 = 0.45 per cent.

*Bladder urine*, 0.3 cc.: $AgNO_3$, 3.0 cc.: KCNS, 3.0 cc.: Cl = 0.

*Plasma*, 1 cc.: 8 cc. supernatant fluid. $AgNO_3$, 4 cc.: KCNS, 2.2: 1.8 cc. $AgNO_3$ for 0.8 cc. plasma = 0.37: corrected 0.38[3] per cent NaCl.

[3] Corrected for pipette calibrations and sp. gr. of plasma. Before insertion in Table II, this figure was divided by 0.975 to correct for plasma protein (see text).

*Experiment 5.*—May 11. Weight of glomerular urine = 1.38 mg. Four standards: (1) 4 cc.; (2) 5 cc.; (3) 6 cc.; (4) 7 cc. standard NaCl. No. 1 chosen. Set at 30.

*Readings:* (R) 27.1, 25.2, 24.7, 24.5, 25.0, 24.8, 26.6, 24.5.
Average 25.3 = 0.43 per cent.
(W) 26.0, 25.7, 25.0, 25.9, 26.0, 26.6.
Average 25.87 = 0.42 per cent.

*Bladder urine*, 2 cc.: $AgNO_3$, 3.0 cc.: KCNS, 2.9 cc.: 0.1 cc. $AgNO_3$ for 2 cc. urine = 0.008 per cent NaCl.

*Plasma*, 1 cc.: 8 cc. supernatant fluid: $AgNO_3$, 4 cc.: KCNS, 2.5 cc.: 1.5 cc. $AgNO_3$ for 0.8 cc. plasma = 0.31: corrected, 0.32 per cent NaCl.

*Experiment 6.*—May 14. Weight of glomerular urine = 1.07 mg. Four standards: (1) 3 cc.; (2) 3.5 cc.; (3) 4 cc.; (4) 5 cc. No. 1 chosen. Set at 30.

*Readings:* (W) 32.4, 32.0, 31.6, 32.6, 31.0, 32.0, 30.9, 31.6.
Average 31.76 = 0.33 per cent.
(R) 36.1, 35.2, 32.7, 33.8, 32.6, 31.5, 32.3.
Average 33.46 = 0.32 per cent.

*Plasma*, 0.55 cc.: 4 cc. of supernatent fluid taken. 0.7 cc. $AgNO_3$ = 0.29 per cent: corrected, 0.30 per cent NaCl.

*Bladder urine* not taken.

*Experiment 7.*—May 16. Weight of glomerular urine = 1.12 mg. Four standards: (1) 2.5 cc.; (2) 3.0 cc.; (3) 3.5 cc.; (4) 4.0 cc. No. 4 chosen = 0.005 mg. NaCl. Set at 30.

*Readings:* (W) 34.6, 35.8, 35.2, 36.0, 35.0, 34.9, 35.3, 35.6.
Average 35.3 = 0.38 per cent.
(R) 35.4, 36.3, 34.2, 34.2, 36.6, 34.7, 34.3, 34.1.
Average 34.97 = 0.38 per cent.

*Plasma*, lost.

*Bladder urine*, 0.8 cc. = 0.4 cc. $AgNO_3$ = 0.082 per cent NaCl.

*Experiment 8.*—May 17. Weight of glomerular urine = 1.04 mg. Five standards: (1) 4 cc.; (2) 5 cc.; (3) 6 cc.; (4) 7 cc.; (5) 8 cc. No. 5 chosen = 0.01 mg. set at 30.

*Readings:* (W) 23.8, 24.0, 23.5, 24.3, 24.4, 24.5, 24.5, 24.2.
Average 24.15 = 1.19 per cent.
(R) 23.0, 23.5, 23.4, 23.4, 24.2, 23.1, 23.5, 23.3.
Average 23.425 = 1.23 per cent.

*Plasma*, 0.7 cc.: 3.5 cc. supernatent fluid taken = 2.43 cc. $AgNO_3$ = 1.14: corrected = 1.18 per cent NaCl.

*Bladder urine*, (weighed) 0.209 gm. = 1.3 cc. $AgNO_3$ = 1.02 per cent NaCl.

*Experiment 9.*—May 18. Glomerular urine = 2.84 mg. Four standards: (1) 6 cc.; (2) 7 cc.; (3) 8 cc.; (4) 10 cc. No. 4 chosen = 0.0125 mg.; 30 mm. (This glomerular urine was made up in 50 cc. flask + 4 cc. $HNO_3$).

*Readings:* (W) 27.0, 28.0, 27.0, 27.1, 27.0, 27.3, 27.2, 27.6.
Average 27.27 = 0.97 per cent.

(R) 26.0, 26.0, 27.0, 27.7, 26.0, 26.3, 26.7, 25.6, 26.0.
Average 26.37 = 1.00 per cent.

*Plasma*, 1 cc.: 8 cc. filtrate: 2.23 cc. $AgNO_3$ = 0.46: corrected, 0.47 per cent NaCl.

*Bladder urine*, 0.53 cc.: 0.85 cc. $AgNO_3$ = 0.264 per cent NaCl.

*Experiment 10.*—May 21. Weight of glomerular urine = 1.36 mg. Standard, 10 cc. = 0.0125 mg. NaCl. Set at 30.

*Readings:* (W) 33.0, 32.8, 32.0, 32.4, 33.0, 32.6, 33.0, 33.0, 32.2.
Average 32.67 = 0.84 per cent NaCl.

(R) 33.2, 32.2, 32.2, 32.6, 32.2, 32.2, 31.8, 32.1.
Average 32.31 = 0.85 per cent NaCl.

*Plasma*, 1 cc.: 8.5 cc. filtrate: 3.3 cc. $AgNO_3$ = 0.64: corrected, 0.66 per cent NaCl.

*Bladder urine*, 1 cc.: 1.65 cc. $AgNO_3$ = 0.27 per cent NaCl.

*Experiment 11.*—May 31. Glomerular urine: 2.01 mg. Four standards: (1) 4 cc.; (2) 5 cc.; (3) 6 cc.; (4) 7 cc. No. 2 chosen = 0.00625 mg. Set at 30 mm.

*Readings:* (W) 34.6, 34.0, 35.2, 33.9, 35.0, 34.4, 35.2, 34.8.
Average 34.64 = 0.54 per cent.

(R) 34.6, 33.7, 34.7, 34.9, 33.2, 34.5.
Average 34.27 = 0.54 per cent.

*Plasma* of composite sample, 0.83 cc.: 7 cc. supernatant fluid: 1.89 cc. $AgNO_3$ = 0.45: corrected, 0.46 per cent NaCl.

*Plasma* of blood taken at end of experiment, 1 cc.: supernatant fluid, 8 cc.: $AgNO_3$, 2.19 cc. = 0.45: corrected 0.46 per cent: NaCl.

No *bladder urine*.

*Experiment 12.*—June 1. Glomerular urine = 2.45 mg. Standard = 7 cc. NaCl. = 0.00875 mg. NaCl. Set at 30 mm.

*Readings:* (W) 33.4, 32.4, 32.6, 33.2, 34.0, 32.4, 33.0, 33.0.
Average 33.0 = 0.33 per cent.

(R) 32.2, 32.2, 32.1, 32.1, 32.2, 32.0.
Average 32.13 = 0.33 per cent.

*Plasma* of composite sample, 0.75 cc.: 6 cc. supernatant fluid: 1.08 cc. $AgNO_3$ = 0.30: corrected, 0.30 per cent NaCl.

*Plasma* from blood taken at end of experiment, 1 cc.: supernatant fluid, 8 cc.: $AgNO_3$, 1.4 cc. = 0.29: corrected, 0.30 per cent NaCl.

*Bladder urine* at end of experiment, Cl = 0.

*Experiment 13.*—June 2. Minute bubbles of mercury transferred from collecting pipette to capillary weighing tube. Hence a second capillary weighing tube was prepared and the uncontaminated part of the glomerular urine transferred to it. Weight of urine (a fraction only of that collected) = 1.19 mg. Standard = 4 cc. NaCl = 0.005 mg. NaCl. Set at 30.

*Readings:* (W) 28.6, 29.0, 29.0, 29.8, 29.9, 29.2, 30.0, 29.7.
Average 29.4 = 0.43 per cent.

(R) 29.1, 28.7, 29.6, 30.0, 29.0, 28.9.
Average 29.22 = 0.43 per cent.

*Plasma* of composite sample, 0.65 cc.: 5 cc. supernatant fluid required 0.9 cc. $AgNO_3$: 0.30: corrected, 0.30 per cent NaCl.

*Plasma* from blood taken at end of experiment, 0.85 cc.: supernatant fluid, 7 cc.: $AgNO_3$, 1.35 cc. = 0.32: corrected, 0.32 per cent NaCl.

No *bladder urine*.

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# QUANTITATIVE ESTIMATION OF MINUTE AMOUNTS OF UREA.

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It is the purpose of this paper to describe an adaptation of existing methods which makes possible the measurement, within probable error of 10 per cent, of amounts of nitrogen in the form of ammonia or urea of the order of 0.0005 mg. The work was undertaken for the purpose of estimating the urea content of the glomerular urine of frogs. At the time when our collaboration ended it had reached a stage at which the accuracy of the method had been tested with satisfactory results on watery solutions of pure ammonium sulfate and of pure urea: and in five experiments it has been used in the analysis of glomerular urine. The figures obtained for urea in glomerular urine in these experiments will not be published until the results can be confirmed and amplified: but since the method is being made the basis of further work in this laboratory and may be of use to others it is thought proper to publish this description.

The essentials of the method consist in hydrolysis of the urea solution by heating with acid at 150°C. (Benedict and Gephart (1); Folin and Wu (2)), simultaneous Nesslerization of the hydrolysis mixture and a known ammonium sulfate solution under identical conditions (Folin and Farmer (3)) and quantitative comparison of the intensities of color produced by the method of Dale and Evans (4).

The water for making solutions and cleaning glassware was prepared by redistilling the usual laboratory distilled water after addition of potassium hydroxide, in the same apparatus and with the same precautions against contaminations as were described in the previous paper (5). The first 3 litres of every distillate were

discarded, and every lot was accepted for use only after the Nessler test, applied in the ordinary way, had shown absence of ammonia. After distillation it was kept in a hard glass container in the dark room, previously described, from which laboratory fumes and dust were restricted. It was always retested before use.

Nessler's reagent was prepared according to the directions of Folin and Wu (2). It was perfectly clear but possessed a faint yellow tinge.

Normal hydrochloric acid, chosen for hydrolysis in preference to sulfuric because it was easier to get free from ammonia, was prepared from the purest obtainable NaCl and $H_2SO_4$. The gas was washed with water and collected in pure water, titrated, and appropriately diluted. Nessler's reagent showed it to be free from ammonia.

Standard ammonium sulfate was prepared according to the directions of Folin and Farmer (3). A stock solution in N/5 HCl ($NH_3$-free) containing 0.1 mg. N in 1 cc. was prepared. For color comparisons, this was diluted fifty times with water.

*Color Comparisons.*—The comparator apparatus described by Dale and Evans was employed. Glass tubes, carefully selected and known to be of the same inside diameter (5 mm.), each 8 cm. long and having one end closed by a flat thin clear glass plate fused on were made for us by the Klett Manufacturing Company of New York. The cut, polished edge of the other end of each tube was carefully coated with black enamel paint in order to get rid of the illuminated ring which otherwise encircles the colored field and is a disturbing element in accurate comparison. An accurate 1 cc. mark was etched on each tube. Illumination was secured by a powerful electric bulb, the rays from which were diffused through a frosted screen of "daylight" glass. For excluding from the eye all light which did not pass through the solutions in the tubes, a rectangular mat was made of thick black rubber tissue through which two circular holes were bored of such diameter that the comparator tubes were tightly held in place after insertion. The mat was somewhat larger than the upper opening of the comparator box and was completely opaque.

In such tests as are summarized in Table I, a measured portion (0.07 to 0.2 cc.) of standard ammonium sulfate solution (1 cc. = 0.002 mg. N) was delivered into each of two comparator tubes,

0.2 cc. of Nessler's solution was added to each, and the contents brought up to the mark with water. After carefully inverting

TABLE I.

*Quantitative Comparisons of Different Portions of the Same Standard Solution of Ammonium Sulfate. One Portion Was Arbitrarily Chosen as a Standard, the Other as Unknown.**

| Date. | Observer. | Nitrogen in standard. | Nitrogen in unknown. | Readings standard: unknown. | Nitrogen found in unknown. | Error. |
|---|---|---|---|---|---|---|
| | | *mg.* | *mg.* | *mm.* | *mg.* | *per cent* |
| June 14 | A. N. R. | 0.0002 | 0.00024 | 61:53 | 0.00023 | −4.2 |
| | J. T. W. | 0.0002 | 0.00024 | 61:51 | 0.000239 | −0.4 |
| | A. N. R. | 0.00016 | 0.0002 | 61:47 | 0.000208 | +4.0 |
| | J. T. W. | 0.00016 | 0.0002 | 61:47 | 0.000208 | +4.0 |
| | | 0.0004 | 0.00032 | 47.0:60.5 | 0.000311 | −2.8 |
| | | | | 48.0:60.5 | 0.000317 | −1.0 |
| | | | | 48.0:60.5 | 0.000317 | −1.0 |
| June 16 | J. T. W. | 0.0002 | 0.00014 | 47.5:60.5 | 0.000157 | +12.1 |
| | | | | 47.0:60.5 | 0.000155 | +10.7 |
| | | | | 46.0:60.5 | 0 000152 | +8.6 |
| | | | | 46.0:60.5 | 0 000152 | +8.6 |
| | A. N. R. | 0.0002 | 0.00026 | 60.5:43.5 | 0.000278 | +6.9 |
| | | | | 60.5:46.5 | 0.000260 | 0 |
| | | | | 60.5:48.5 | 0.000249 | −4.2 |
| | | | | 60.5:46.5 | 0.000260 | 0 |
| | | 0.00016 | 0.00018 | 60.5:56.5 | 0.000171 | −5.0 |
| | | | | 60.5:51.5 | 0.000188 | +4.4 |
| | | | | 60.5:53.5 | 0.000183 | +1.7 |
| | | | | 60.5:53.5 | 0.000183 | +1.7 |
| Oct. 11† | A. N. R. | | 0.00028 | | 0.000276 | −1.4 |
| | | | 0.00022 | | 0.000224 | +1.8 |
| | | | 0.00030 | | 0.000262 | −12.7 |
| | | | 0.00038 | | 0.000392 | +3.2 |
| Oct. 12 | A. N. R. | | 0.00024 | | 0.000248 | +3.3 |
| | | | 0.00026 | | 0.000266 | +2.3 |
| | | | 0.00041 | | 0.000384 | −6.3 |
| | | | 0.000282 | | 0.000266 | −5.7 |
| | | | 0.000254 | | 0.000234 | −7.9 |
| | | | 0.000254 | | 0.000272 | +7.1 |

* In all except two of these estimations the observer was ignorant of the strength of either solution.

† Complete data of this and the following tests are not now available. The correctness of the figures given is vouched for.

the tubes several times to secure uniform mixture, they were inserted into the holes in the mat of the comparator apparatus.

After deciding in which tube the color was more intense, fluid was removed from this by means of a clean pipette drawn out to a slender long point and fitted at the other end with a small rubber bulb. After practice, by successively withdrawing and restoring fluid, identity of intensity of color in the two tubes could be satisfactorily attained. The height of fluid column in each tube was then measured with a steel rule graduated in half millimeters and the numerical expression of the relationship of the contents was calculated in the usual way. The color comparisons were all made in the small dark room.

For applying the method to minute amounts of urea solutions, the following procedure was adopted.

Baker's urea, twice recrystallized from alcohol and dried at room temperature *in vacuo*, was used in preparing a solution of such strength that 1 cc. = 0.2 mg. N. The strength of the mother solution from which this dilution was made was carefully determined by Dr. A. K. Balls by the Kjeldahl method. A small amount (1 to 3 mg.) of this solution was drawn up into the capillary pipette used for collecting glomerular urine and discharged into a weighed capillary tube in the manner described in the previous paper (5).[1] After weighing the tube with its contents, the fluid was transferred[2] to a perfectly clean, dry, hard glass tube (pyrex) open at one end, 5 mm. inside diameter and 6 inches long. The tube was provided with an accurate 1 cc. mark. Enough normal HCl, accurately measured, was then introduced into the tube to make the acid concentration of the contents approximately 0.1 N. After sealing the upper end of the tube in an oxygen flame it was autoclaved at 150°C. for 1 hour. On removal from the autoclave, the top of the tube was broken off and the inner surface of the top rinsed with a few drops of water into the main bulk of the fluid in the larger fraction of the tube.

A series of the small comparator tubes, which have been described, perfectly clean and dry, were then charged with different amounts of the standard ammonium sulfate solution (1 cc. = 0.002 mg. N), the range of quantities chosen (0.1 to 0.3 cc.) being such as to include an amount of nitrogen of the same order as that which was anticipated in the urea solution. Normal hydrochloric

[1] Wearn and Richards (5), p. 259.
[2] Wearn and Richards (5), p. 261.

TABLE II.

*Estimations of Nitrogen in Weighed Samples of a Known Solution of Urea (1 Cc. = 0.2 Mg. N).*

| Date. | Weight of sample. | Obeerver. | Nitrogen in standard. | Nitrogen in sample. | Readings standard: sample. | Nitrogen found. | Error. |
|---|---|---|---|---|---|---|---|
| | *mg.* | | *mg.* | *mg.* | *mm.* | *mg.* | *per cent* |
| June 29 | 2.46 | J. T. W. | 0.0005 | 0.000492 | 62.5:58.5 | 0.000534 | +8.4 |
| | | A. N. R. | | | 62.5:61.5 | 0.000508 | +3.2 |
| July 2 | 1.67 | | 0.00032 | 0.000334 | 61:59.5 | 0.000328 | −1.8 |
| Oct. 12 | 2.45 | J. T. W. | 0.0006 | 0.00049 | 42:48 | | |
| | | | | | 42.5:48 | | |
| | | | | | 41:48 | | |
| | | | | | 40:48 | | |
| | | | | Average | 41.37:48 | 0.000517 | +5.5 |
| | | A. N. R. | | | 39.5:48 | | |
| | | | | | 41.5:48 | | |
| | | | | | 40.5:48 | | |
| | | | | Average | 40.5:48 | 0.000506 | +3.3 |
| | 2.3 | J. T. W. | 0.0005 | 0.00046 | 39.5:48 | | |
| | | | | | 41.5:48 | | |
| | | | | | 40.5:48 | | |
| | | | | Average | 40.5:48 | 0.00042 | −8.7 |
| | | A. N. R. | | | 41:48 | | |
| | | | | | 41.5:48 | | |
| | | | | | 41.5:48 | | |
| | | | | Average | 41.3:48 | 0.00043 | −6.5 |
| | | J. T. W. | 0.0004 | | 51:49 | | |
| | | | | | 51:49 | 0.00042 | −8.7 |
| | | A. N. R. | | | 51:49 | | |
| | | | | | 51:46 5 | | |
| | | | | Average | 51:47.75 | 0.00043 | −6.5 |

acid was than added in such amount as to make the total acid added to each exactly equal to that added to the urea solution. Then 0.2 cc. of Nessler's solution was added to the contents of each standard tube and also to the hydrolyzed urea solution. The volume of the contents of each tube was made up exactly to the 1 cc. mark and thoroughly mixed. The Nesslerized urea-hydrolysis mixture was transferred by means of a clean, dry, long pointed pipette to a clean, dry comparator tube. The standard tube which most closely resembled the urea tube in intensity of color was chosen, the two tubes inserted into the mat of the comparator apparatus, and quantitative equalization of color made in the manner described.

Results of such estimations are summarized in Table II.

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# CARBOHYDRATE UTILIZATION.

## I. RATE OF DISAPPEARANCE OF *d*-GLUCOSE FROM THE BLOOD.

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Although the disappearance of *d*-glucose from the blood has been used as the basis of many clinical investigations for the testing of the tolerance of the body for glucose, very little is really known of the factors involved in this mechanism of regulating and maintaining the blood sugar level.

The alimentary sugar tolerance test depends on giving a definite amount of *d*-glucose per kilo body weight and on determining the blood sugar concentration before the test and at certain intervals thereafter. Normally the blood sugar rises to a maximum at about the ½ hour period and returns to the normal level within 1 to 2 hours.

It has been shown by a number of investigators that the fall in the blood sugar is not due to absorption. Johansson (1), by x-ray examination, gave evidence that the absorption of 200 gm. of glucose is not complete until after about 4 hours. Indirect evidence was obtained by Janney and Isaacson (2) that absorption was not a factor in their experiments. They followed the excretion of nitrogen after the feeding of meat to their dogs before and after thyroidectomy in which they found a decreased tolerance. MacLean and de Wesselow (3) found that additional large doses of sugar given during the height of absorption failed to raise the blood sugar above the previous level. Furthermore, these same investigators, as well as Foster (4), Hamman and Hirschman (5), and others have found that a second dose of sugar, given after the return of the blood sugar level to normal from the effects of the first dose, gives little if any rise in the blood sugar value. Our own results support this.

When the glucose is introduced into the alimentary canal, it is readily absorbed from the gut, thence passing into the blood causing an increase in the blood sugar concentration. At some time during the rise of the blood

sugar a mechanism is stimulated that soon takes the sugar out of the blood at a faster rate than it is being absorbed, bringing the blood sugar level back to normal or even slightly below normal. By tolerance for sugar, in this paper, we mean the ability of the organism to remove this excess sugar from the blood, or in other words the rate of disappearance of the sugar from the blood. We are not interested in the amount of sugar excreted in the urine. MacLean and de Wesselow (3) have put forth the hypothesis that it is the presence of an excess of sugar in the circulation that stimulates the mechanism that deals with sugar. This is strongly supported by Foster (4). Although sugar must stimulate this mechanism as shown by the successive tolerances, there must be some other factors than the concentration of the blood sugar itself, as we shall show later. Greenwald (6), although agreeing with the excess sugar stimulation hypothesis in most part, nevertheless felt that hyperglycemia itself could not explain the results he obtained in the lowering of sugar tolerance by fat diets and not by proteins. There seems no doubt, though, that there is some mechanism that is dormant during the first part of the tolerance test, which is later stimulated to action.

What this mechanism is, we can only hypothecate for the present. We do know though that the glycogenetic function of the liver and muscles plays a major part, and that the endocrine secretions are intimately concerned.

Foster (4) has suggested that the ingestion of glucose or the hyperglycemia that ensues stimulates the pancreas to put out its internal secretion which then operates in some way to accelerate both glycogen formation and oxidation of sugar. This investigator further points out that the muscle tissues are more important in connection with glycogen storage than is perhaps generally believed. Evidence for this is based on the great difference between the sugar concentration of the arterial and venous blood after the ingestion of glucose. Cori and Cori (7), Frank, Nothmann, and Wagner (8), and Lawrence (9), have also found a greater difference between arterial and venous blood during the action of insulin. These facts emphasize the importance of the muscles and tissues in the mechanism of taking sugar out of the blood. Mann and Magath (10) have shown that the liver is not concerned with the fall in blood sugar due to insulin but that it is necessary for the return to normal.

We generally speak of the glycogen formation in connection with the disappearance of sugar from the blood. However, there are many indications that this disappearance of the sugar is due to the formation of substances other than glycogen. Macleod (11) states in his review on insulin, ". . . . the outstanding facts are that insulin causes the sugar of the blood (and tissues) to diminish both in the normal and diabetic animal, partly because of the increase in the relative amount of carbohydrate that is metabolized and partly because some of the glucose is converted into non-saccharine material, which is mainly glycogen in diabetes, but is some other substance in the normal animal." Palmer (12) came to the conclusion that there are present in muscle tissue higher sugars or polysaccharides of which maltose probably constituted the larger

part. Since phosphorus has been found to decrease in the blood after the injection of insulin, it is highly indicative that the substance formed might be a phosphoric acid complex (6, 13–16). Although they found that there was a fall in the organic phosphates of the blood after insulin, Blatherwick, Bell, and Hill (13) did not find that the ingestion of glucose itself had any definite effect on the phosphates of the blood. This tends to support the idea that the process of taking excess sugar from the blood normally is different in principle from the effect of injected insulin, a point not in accord with the hypothesis of excess sugar in the blood stimulating the pancreas. However, Harrop and Benedict (14) claim a decrease in phosphates after the ingestion of glucose. Besides confirming the latter, Bolliger and Hartman (17) have presented curves showing that the phosphate level remains very constant in diabetes after the ingestion of glucose but that under the influence of insulin the curve behaves normally, showing a drop followed by a rise to normal. Since Cori and Goltz (18) have demonstrated that the decrease of the free sugar of the liver is not due to the formation of a phosphate complex, our attention is once more directed to the muscles as the seat of this possible synthesis probably influenced by insulin.

Evidence pointing to the connection of the endocrines with sugar metabolism has been obtained both experimentally and clinically. The sugar tolerance curve has been used in many disorders. It has been used in diabetes, hyper- and hypothyroidism, hyper- and hypopituitarism, Addison's disease, cancer, arthritis, tuberculosis, mental diseases, infection, anemia, nephritis, and cirrhosis of the liver. Consideration of the behavior of these curves, especially in endocrine disorders, may throw some light on the part played by these glands normally or at least indicate whether or not they are connected with the sugar utilization mechanism.

We have already considered the rôle of the pancreas from the standpoint of insulin. The high and prolonged curve in diabetes again points to the importance of the pancreas in the ability of the organism to remove excess sugar from the blood.

Consistent results have also been found in hyperthyroidism; namely, an abnormally high curve indicating a reduced tolerance (19–23). Labbé, Labbé, and Nepveux (24) suggest the test as a means of differentiating between simple goiter and Basedow's disease, the former giving little if any hyperglycemia while the latter gives a very marked hyperglycemia after the ingestion of 45 gm. of glucose. The reduced tolerance is not connected with the oxidation of sugar as shown by respiratory quotient studies (25). An increased diastatic activity of the blood besides the decreased tolerance has been found in hyperthyroid cases by Killian (23), while in hypothyroid cases the reverse was found. This seems to indicate that the thyroid secretion acts by increasing glycogenolysis rather than by decreasing or inhibiting glycogenesis. Also, Janney (2) has shown that thyroidectomy results in a distinct hypoglycemia. A similar fact was observed by Bodansky, Simpson, and Goldberg (26) in thyroidectomized sheep. McCurdy (27) found that removal of the thyroid

raises the assimilation limit for glucose. Furthermore, a great many observations have been made in recent years to show that there is an increased sugar tolerance in myxedema (19, 28). Kuriyama (29) found that feeding thyroid to white rats produced a decreased glycogen content of the liver. He did not find, however, that experimental hyperthyroidism produced a change in sugar content of the blood in either rats or rabbits. This evidence rather points, although not necessarily, to an inhibition of glycogenesis rather than increased glycogenolysis. Nevertheless, it is certain that disturbances of the thyroid do cause disturbances in the mechanism controlling sugar metabolism, but it is still an open question whether this is done through the thyroid itself or indirectly through an effect on the pancreas or on the nervous system.

The relationship between the parathyroid glands and carbohydrate metabolism is not clear, the evidence being somewhat conflicting. In general it is assumed that there is an increased mobilization of carbohydrates in tetany which is considered to be due to decreased parathyroid activity (30).

Cushing (31) has demonstrated the importance of the hypophysis in carbohydrate metabolism. Administration of the posterior lobe extract lowered the sugar tolerance to normal or below normal in animals in which it had been raised by excision of the posterior lobe. The decreased tolerance has been observed frequently in acromegaly. On the other hand, a hypoglycemia and an increased tolerance have been associated with hypopituitarism (19, 28). In dystrophia adiposogenitalis, the carbohydrate tolerance is increased; sometimes it is almost unlimited (32). According to Cushing both the increased sugar tolerance and hypoglycemia can be remedied by injection of posterior lobe extract. Cushing believes that the posterior lobe of the hypophysis has a glycogenolytic action. Furthermore it has been shown that the simultaneous injection of the posterior lobe extract enhances the hyperglycemia induced by intravenous injection of glucose (33). It has also been found that pituitrin causes a rise in blood sugar concentration (34). Kojima obtained changes in the pancreas as the result of feeding pituitary gland (35). However, it must be borne in mind that in hypopituitarism and also in hypothyroidism there is an abnormal fat formation which may in part account for the increased tolerance.

It is generally accepted that adrenalin, the secretion of the adrenal glands, exerts a strong stimulation on the glycogenolytic function of the body causing a hyperglycemia. In Addison's disease, which is considered to be the result of a hypofunction of the adrenals, there is present a hypoglycemia and the tolerance for glucose is raised (23, 28). Achard, Ribot, and Binet (33) found that the simultaneous injection of adrenalin and glucose produced a greater hyperglycemia than the sum of the increases obtained from the injection of glucose and adrenalin separately. This would indicate that adrenalin prevents the storage of sugar since it has also been shown by Lusk and Riche (36) that adrenalin does not affect the oxidation of glucose.

Tsubura (37) has reported that castration does not lower the fasting blood sugar but that the tolerance for glucose was lowered.

From time to time references have been made in the literature as to the effect of diet and fasting on the carbohydrate tolerance of the body. Kageura (38) found that the hyperglycemia resulting from the ingestion of the Sakeguchi test meal consisting of 100 gm. of rice and two eggs was greater after a carbohydrate-poor diet than after one rich in carbohydrates. He claimed that a protein diet was equally effective in lowering the tolerance to glucose as a fat diet (39). However, as pointed out by Greenwald (6), his protein diet consisted largely of fat. Greenwald found that in both man and dog a fat diet lowered the tolerance to glucose but that a protein diet did not produce this effect, in fact, it had a beneficial rather than detrimental effect. It was observed by Southwood (40) that an increased hyperglycemia resulted in men who had been on a carbohydrate-free diet for 36 hours before the test. Staub (41) found that the hyperglycemic reaction after the ingestion of 20 gm. of glucose was less after a fast of 10 to 15 hours than after one of 5 hours, but that it became more marked after a fast of 24 hours. An increased hyperglycemia was obtained after 2 days on a carbohydrate-free diet and also after severe work. During the work the hyperglycemia was less than at rest. Similar results have been reported by Traugott (42) who found that the ingestion of 20 to 100 gm. of glucose in a normal well nourished man produced a hyperglycemia of 30 to 50 per cent while after 3 days starvation it produced a hyperglycemia of 243 per cent.

We have found in rabbits that fasting greatly decreases the rate of disappearance of glucose from the blood after a hyperglycemia. We have endeavored to ascertain the cause of this decrease and what substances would bring the tolerance back to normal, in other words what stimulates the mechanism for taking the sugar out of the blood. By this we hoped light might possibly be thrown on the process involved in the regulation of the blood sugar level and removal of glucose from the blood.

## *Methods.*

The rabbits used in these experiments were bled from the marginal ear vein. About 1 cc. of blood was taken for the determination of the blood sugar which was done by the method of Folin and Wu (43); for the determination of pH the blood was collected from the marginal vein of the ear. The blood was brought to the ear by applying xylol freely. The vein was cut and the blood allowed to collect on the ear in a little pool covered with paraffin oil and the blood pipetted from this. The pH was determined by the method of Hawkins (44). The carbon dioxide capacity was found by the method of Van Slyke and Stadie (45).

In the alimentary glucose tolerance tests the rabbits were given 3 gm. of pure *d*-glucose per kilo of body weight in 35 cc. of water. The blood sugar was determined at ½ hour intervals for

1½ hours and then at hourly intervals for 2 hours. In the intravenous tolerances 2 cc. of 50 per cent solution of *d*-glucose per kilo were injected into the marginal vein of the ear, and the blood sugar determined at intervals of ½ hour.

In cases where the tolerance test was made after fasting the dosage of sugar was based on the weight of the animal before fasting. During the fasting periods the rabbits had water *ad libitum.*

TABLE I.

*Alimentary Glucose Tolerance after 1 Day Fast.*

3 gm. glucose per kilo.

| Rabbit No. | Normal blood sugar per 100 cc. | Periods after ingestion of glucose. | | | | |
|---|---|---|---|---|---|---|
| | | ½ hr. | 1 hr. | 1½ hrs. | 2½ hrs. | 3½ hrs. |
| | *mg.* | | | | | |
| 17 | 107 | 165 | 163 | 142 | 129 | 127 |
| 1 | 100 | 170 | 190 | 165 | 130 | 108 |
| 3 | 125 | 190 | 230 | 200 | 125 | 120 |
| 4 | 110 | 170 | 180 | 165 | 130 | 120 |
| 5 | 125 | 165 | 175 | 175 | 160 | 135 |
| 7 | 135 | 190 | 200 | 170 | 140 | 120 |
| 8 | 135 | 175 | 180 | 170 | 140 | 125 |
| 10 | 130 | 171 | 209 | 170 | 142 | 130 |
| 21 | 105 | 179 | 165 | 137 | 129 | 110 |
| 23 | 133 | 190 | 178 | 165 | 134 | 122 |
| 32 | 125 | 185 | 200 | 175 | 140 | 128 |
| 11 | 135 | 260 | 210 | 170 | 155 | 130 |
| Average... | 122 | 184 | 190 | 167 | 138 | 123 |

EXPERIMENTAL.

*Effect of Fasting on Sugar Tolerance.*

Table I gives the results of feeding 3 gm. of glucose per kilo to twelve rabbits that had been fasted 1 day. Table II presents the results of similar tests on nine rabbits that had been fasted for 4 days. The points of interest in these curves are, the height of the curve, the time at which it returns to normal, and especially the time that the maximum of the curve appears. In the case of the 1 day fast curves, the majority have their maximum at the ½ hour point and have generally returned to normal within 2½ hours. On the other hand, in the 4 day fasted animals the

maximum comes later, in the majority at the 1½ hour point, and it is considerably higher. There is also a decided delay in the return of the blood sugar concentration to the normal level. At 3½ hours after the ingestion of the glucose the blood sugar is still elevated. The composite curves in Chart 1 bring these points out clearly. In no case has there been a failure to find a decreased tolerance after fasting. The two rabbits with no fast were taken from the pen after having eaten ground grain and bread for 2 to 3 hours *ad libitum*. The tolerance test was done about ½ hour later. The utilization of the ingested glucose

TABLE II.

*Alimentary Glucose Tolerance after 4 Day Fast.*

3 gm. glucose per kilo.

| Rabbit No. | Normal sugar per 100 cc. | Periods after ingestion of glucose. | | | | |
|---|---|---|---|---|---|---|
| | | ½ hr. | 1 hr. | 1½ hrs. | 2½ hrs. | 3½ hrs. |
| | *mg.* | | | | | |
| 17 | 105 | | 185 | 195 | 192 | 187 |
| 24 | 123 | | 210 | 235 | 200 | 160 |
| 27 | 113 | 188 | 200 | 190 | 180 | |
| 28 | 115 | 173 | 182 | 186 | 195 | |
| 32 | 123 | | 215 | 215 | 205 | 189 |
| 35 | 150 | | 255 | 302 | 284 | 240 |
| 37 | 132 | 182 | 232 | 258 | 250 | 220 |
| 38 | 130 | 225 | 265 | 270 | 210 | 155 |
| 39 | 123 | | 284 | 292 | 265 | 216 |
| Average... | 123 | 192 | 225 | 238 | 220 | 195 |

was much better than that of the fasted animals and as we will show later was evidently due to the stimulation of the metabolism by the previous carbohydrate diet.

The effect on the glucose tolerance of separate 1 to 4 day fasts on the same animal is shown in Chart 2. These curves show the gradual decrease in tolerance that is brought about by fasting. This rabbit was fasted 4 days, the tolerance test performed, and the rabbit put back on food for 2 days. After this rest period it was then fasted for 3 days, the tolerance test made, and the animal was put back on food again. In the same way tests were carried out after a 2 day fast and then after a 1 day fast. The results show a gradual decrease in tolerance with the in-

crease in the fasting period. This is marked by the height of the curve and the shifting of the maximum point from the ½ hour period to a later one and a delayed return to normal. If loss of weight in itself or the handling of the animals tended to decrease the tolerance, then in this series the tolerance after the

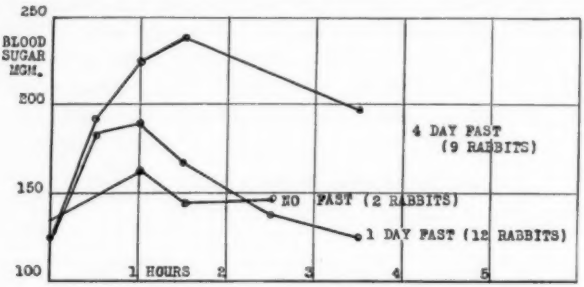


CHART 1. Composite curves.

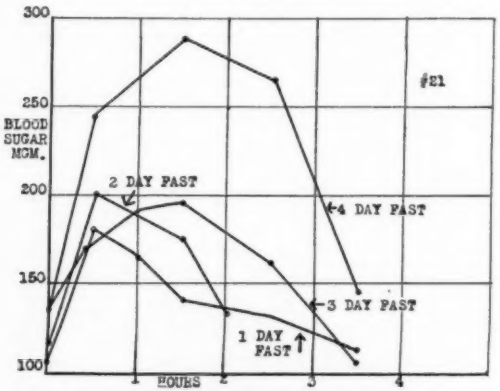


CHART 2. Effect of fasting.

1 day fast should have been less than the one after the 4 day fast. The 4 day test was made first, while the 1 day test was made last.

Further evidence for this contention is given by the results on rabbit No. 17 (Chart 3). This one was fasted 19 days, decreasing in weight from 2250 to 1550 gm. After the tolerance test was performed it was put back on food for 3 days, during which time it increased in weight to 1660 gm. It was then

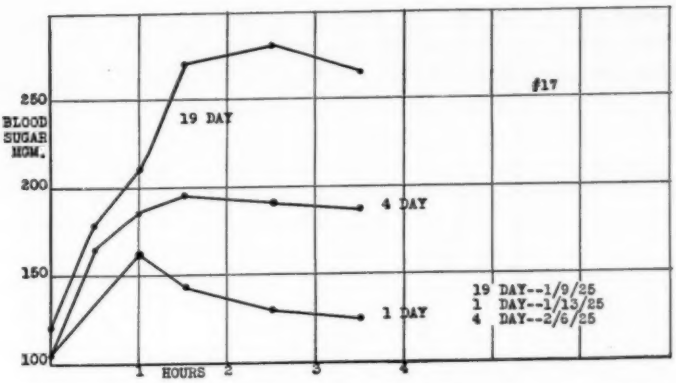


CHART 3. Effect of fasting.

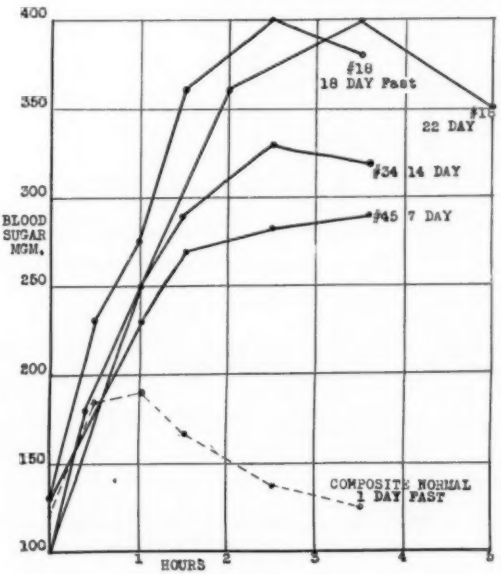


CHART 4. Effect of fasting.

fasted for 1 day and the sugar tolerance repeated. About 3 weeks later, a tolerance test was made after a 4 day fast. At this time it weighed 1770 gm. before and 1570 gm. after the fast.

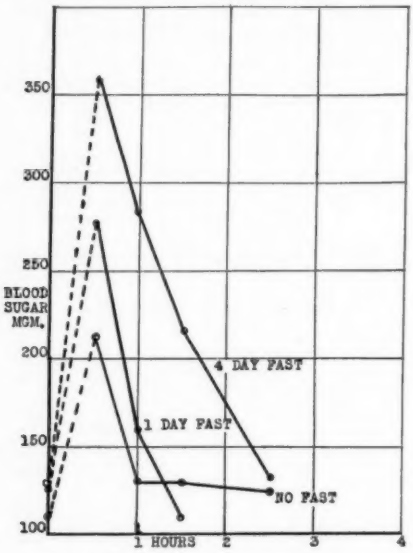


CHART 5. Composite curves. Intravenous glucose.

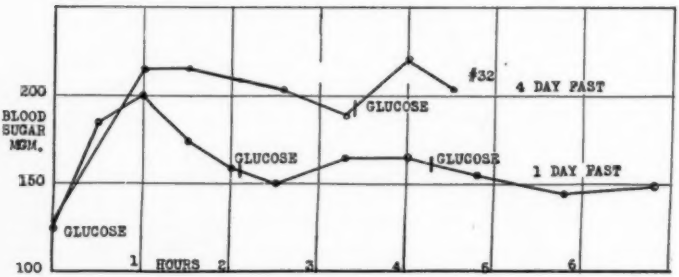


CHART 6. Successive tolerances.

The effects of longer periods of fasting are shown in Chart 4. Here the rate of disappearance of sugar from the blood is greatly reduced.

When glucose was introduced intravenously, its rate of disappearance from the blood stream was decreased by fasting. This offers very definite evidence that in the decreased tolerance caused by fasting we are dealing with the rate of disappearance of glucose from the blood and not with the absorption of glucose from the intestine. The composite curves of the intravenous tolerance tests made after a 1 day fast, a 4 day fast, and after no fast at all, are shown in Chart 5.

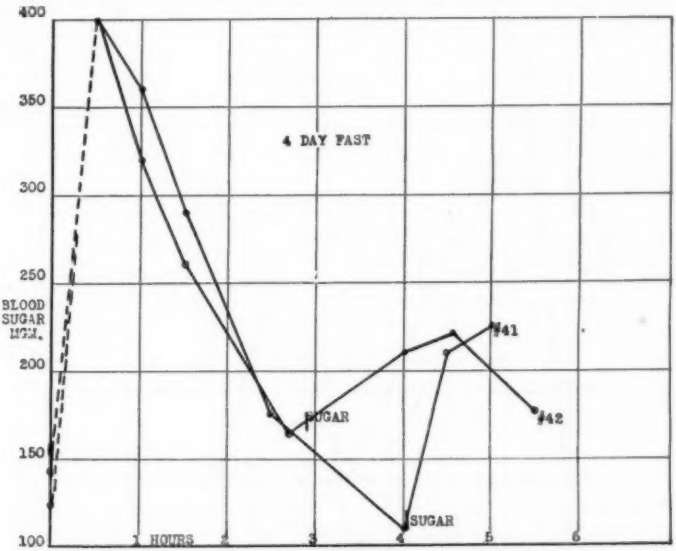


CHART 7. Alimentary tolerance following intravenous tolerance.

The 4 day fast period was selected for the studies on the effect of various substances on the sugar tolerance. It is a fast of sufficient duration to produce a definite and typical change, and yet not too long to make it difficult for the animal to recover from the effects of the inanition.

*Successive Tolerances.*

The results obtained by giving a second dose of sugar, at a time when the blood sugar concentration was on the downward

trend from the height caused by the first dose, agree with those of Foster (4) in that little if any rise occurred as a result of this second dose. Three successive tolerances were tried, even the third dose produced no further rise. However, in this same rabbit No. 32 (Chart 6), after a 4 day fast, a second dose of sugar given at a point where the sugar concentration was falling from the height caused by the first produced a second rise to a point a little higher than the maximum caused by the first. In other words, after a 4 day fast, the first dose of sugar is not as

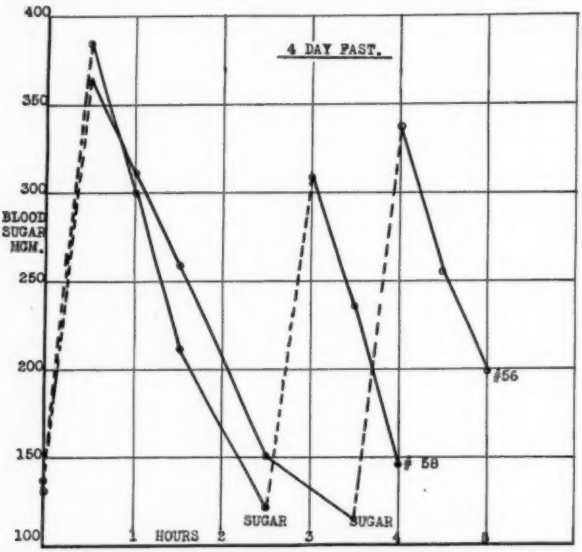


CHART 8. Two successive intravenous tolerances.

effective in stimulating that mechanism which removes the excess blood sugar.

In Chart 7 are presented alimentary tolerance tests following intravenous tolerances in animals that have been fasted 4 days. Here again there is a lack of noticeable stimulation of the sugar mechanism by hyperglycemia in the 4 day fasted animals. The same is also brought out by two successive intravenous tolerances in Chart 8.

*Effect of Various Foodstuffs on the Glucose Tolerance.*

In this series of experiments to determine the efficacy of various foodstuffs in stimulating the sugar-regulating mechanism, the animals were fasted for 4 days before the tolerance test except for a specific foodstuff given at a stated interval before the test. Chart 9 compares the results obtained by the feeding

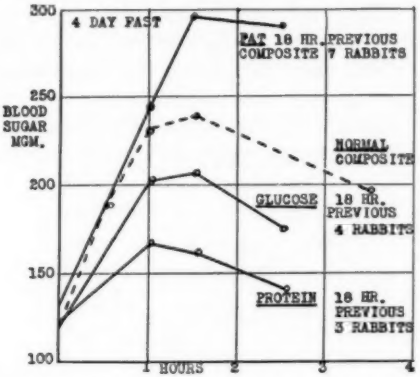


CHART 9. Effect of previous diet.

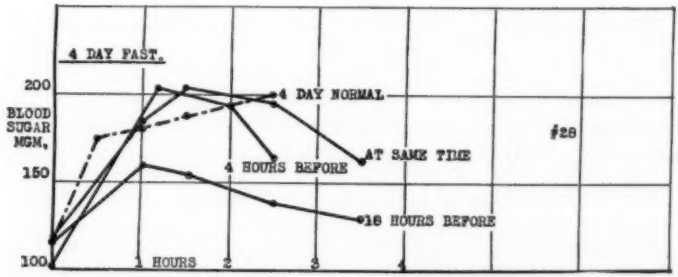


CHART 10. Effect of white of egg with and before glucose.

of fat, glucose, and protein 18 hours before the tolerance test with the normal 4 day fast curve.

The results obtained from feeding 10 gm. of lard oil the night before the test showed a decrease in tolerance.

The effect of glucose was studied by giving 10 gm. of glucose 18 hours before the test. A slight increase in tolerance resulted.

Gelatin seemed to have the greatest effect in increasing the sugar tolerance. 10 gm. given the night before, 18 hours before the test, in all cases produced a remarkable increase in the glucose tolerance.

Chart 10 shows the glucose tolerance tests on a rabbit after a 4 day fast and the effects of 40 gm. of egg white given at the same time, at 4 hours before, and at 18 hours before the glucose was ingested. The protein given simultaneously with the sugar and that given 4 hours before the test had little if any effect on the sugar tolerance, but when given the night before the test resulted in a very greatly increased tolerance.

That the increased tolerance is not related with the presence of material in the alimentary tract is shown by the fact that the

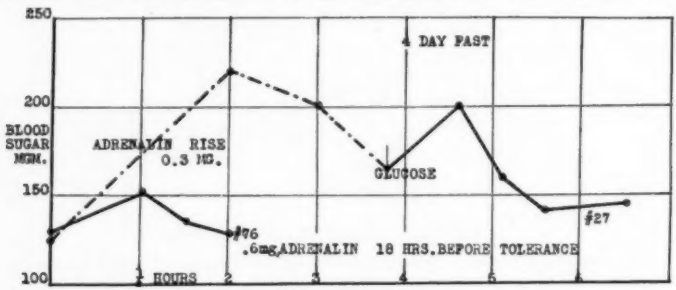


CHART 11. Effect of adrenalin.

fat meal produced no favorable influence on the tolerance; if anything, it decreased the tolerance still further. Furthermore, the feeding of agar-agar produced negative results.

*Effect of Adrenalin and Morphine.*

It is important to determine whether the increasing of the blood sugar concentration from the body's own stores would stimulate the sugar regulatory mechanism. This would also eliminate the possibility of some probable dietary factor, the lack of which, caused by fasting, decreased the tolerance for glucose. Adrenalin was selected for this purpose. When the blood sugar concentration was coming back to normal after the rise caused by the adrenalin, the glucose was given. In the case of rabbit No. 27, as shown in Chart 11, 0.3 mg. of adrenalin

was given hypodermically, causing a rise in the blood sugar from 125 to 220 mg. per 100 cc. in 2 hours. At the $3\frac{3}{4}$ hour point 3 gm. of glucose per kilo were given by stomach tube. The tolerance was only slightly increased, yet more so than a tolerance performed after a previous dose of sugar in an animal fasted 4 days. However, when the adrenalin was given the night before, the tolerance was increased to a remarkable degree (Chart 11).

In considering why adrenalin should be so effective in increasing the tolerance, the rise in the blood sugar suggests itself as being the causative factor. Consequently a hyperglycemia was produced by other means to see if this also would increase

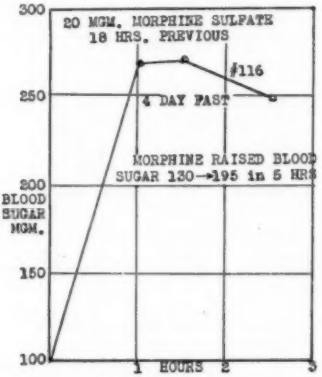


CHART 12. Effect of morphine.

the tolerance. 20 mg. of morphine sulfate were given to the fasted animal the night before the test. Even though the blood sugar concentration was increased as by adrenalin, there was no increase in the sugar tolerance (Chart 12). Evidently the increase of the blood sugar from its own stores with its subsequent removal does not in itself stimulate the sugar regulatory mechanism. The cause of the action of adrenalin in this respect must be found elsewhere.

*Effect of Insulin.*

Since the action of adrenalin with its resultant hyperglycemia tends to increase the rate of disappearance of the sugar from

the blood, it is important to note the effect of insulin with its resultant hypoglycemia.

This was tried by testing the tolerance when the sugar level

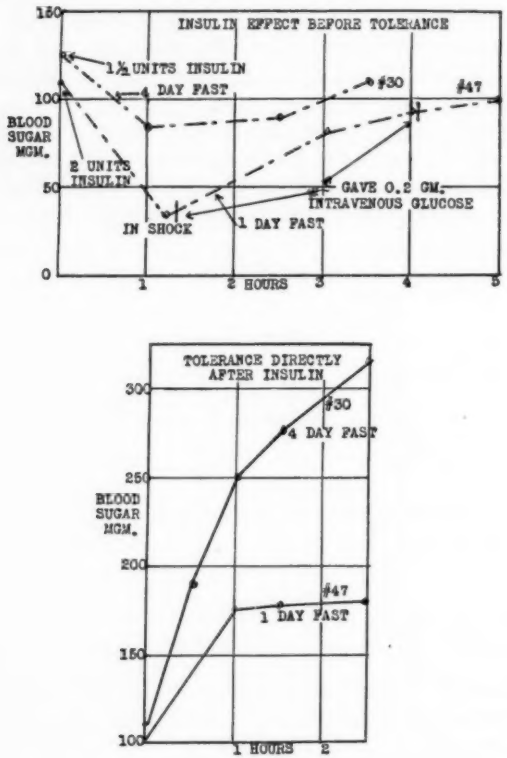


CHART 13. Insulin before tolerance.

had returned to normal after the fall caused by insulin. This was done in both the 1 and the 4 day fasted animals. The results are shown in Chart 13. The tolerance was decreased in both the 4 and the 1 day fasted animals.

*Effect of Sodium Bicarbonate.*

As there is much work that points towards an effect on sugar metabolism by the administration of sodium bicarbonate, it was thought worth while to try the effect of it on the 4 day fasted animals. It was found that the administration of the sodium bicarbonate, when given simultaneously with, a few hours before, or the night before the tolerance test was not as effective in increasing the rate of disappearance of blood sugar as when 1 gm. was administered each day of the fast. In the latter the sugar tolerance at the end of the fasting period was increased similarly to that of an animal after no fast (Chart 14).

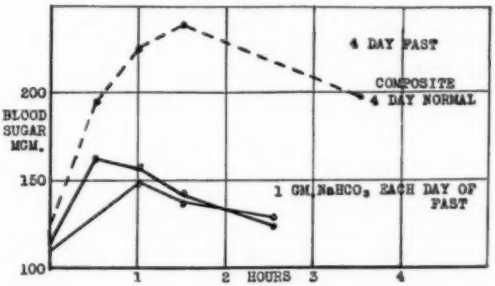


CHART 14. Effect of sodium bicarbonate.

*Effect of Fasting on the pH and $CO_2$ Capacity of Blood.*

The decided effect of the sodium bicarbonate naturally suggests that an acidosis is the cause of the decreased tolerance after fasting. The urine of a number of rabbits fasted from 4 to 7 days gave negative tests for acetone and β-hydroxybutyric acid. It was decided then to study the effect of fasting on the pH and $CO_2$ capacity. As shown in Table III the results show little if any acidosis. Of course this does not preclude the possibility of this decreased tolerance being somehow connected with the acid-base balance of the body. Henderson (46) points out the intimacy between the oxidation of sugar and the blood alkali although he considers the oxidation of the sugar in the tissues as a controlling factor in the regulation of blood alkali. This question of the connection of the acid-base balance of the

blood and the decreased tolerance, also the effect of intermediate metabolites, and other substances are now being studied further in these laboratories.

TABLE III.

*Effect of Fasting on the pH and $CO_2$ Capacity of Blood.*

| Rabbit No. | Determination. | Normal values. | Days of fasting. | | | |
|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 |
| 58 | pH | 7.42 | | | | 7.49 |
| | $CO_2$ capacity. | | | | | |
| | Volume per cent. | 30 | | | | 40 |
| 63 | pH | 7.36 | 7.31 | 7.39 | 7.37 | 7.41 |
| | $CO_2$ | 29 | 25 | 30 | 37 | 35 |
| 80 | pH | 7.36 | | | | 7.38 |
| | $CO_2$ | 32 | | | | 19 |
| 92 | pH | 7.34 | | 7.41 | 7.40 | 7.35 |
| | $CO_2$ | 23 | | 35 | 36 | 27 |
| 86 | pH | 7.39 | 7.40 | 7.40 | 7.38 | 7.38 |
| | $CO_2$ | 39 | 36 | 40 | 41 | 34 |
| 114 | pH | 7.41 | | | | 7.42 |
| | $CO_2$ | 30 | | | | 29 |
| | | | 4 | 7 | 10 | |
| 77 | pH | 7.42 | 7 39 | 7.43 | 7.40 | |
| | $CO_2$ | 38 | 28 | 38 | 29 | |

SUMMARY.

Attention is directed to the metabolic mechanism which is concerned with the rate of disappearance of glucose from the blood after a hyperglycemia.

Further evidence is offered that this mechanism is practically independent of the rate of absorption from the intestine.

Fasting invariably decreases the rate of disappearance of glucose from the blood in a normal rabbit after a hyperglycemia from glucose ingestion.

After a period of fasting, specific foodstuffs vary the rate of disappearance of hyperglycemia. Protein fed with, or 4 hours before, the glucose test meal exerts little or no influence on the blood sugar curve, but given 18 hours previous greatly increases the rate of disappearance. Fat fed 18 hours previous to the glucose test meal decreases the rate. Glucose given 18 hours before the usual glucose test meal slightly increases the rate of disappearance of the sugar from the blood.

Adrenalin given 18 hours previous to the glucose test meal greatly increases the rate of disappearance while morphine does not, although both produce hyperglycemias. This indicates that a previous hyperglycemia is not the only stimulating factor.

A glucose test meal immediately after an insulin hypoglycemia has returned to normal shows a decreased rate of disappearance of sugar from the blood.

Sodium bicarbonate administered during a fast prevents the decrease in rate of disappearance of blood sugar normally occurring after a glucose test meal. Blood pH and $CO_2$ studies indicate that acidosis is not the predominant factor in causing this change during a fast.

The experimental data presented emphasize greatly the importance of carefully controlling the diet, period of fasting, and medication before performing the customary sugar tolerance test used in clinical diagnosis.

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# THE pK′ OF THE HENDERSON-HASSELBALCH EQUATION FOR HYDRION CONCENTRATION OF SERUM.*

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This paper presents the pK′ values for human sera from a series of abnormal conditions. pK′ values were determined at both 38° and 20°. In addition are given values for several normal dog sera.

The Henderson-Hasselbalch equation for expressing the relationship between $CO_2$ tension, $[BHCO_3]$, and pH, has been of first importance in studying the acid-base equilibrium of the blood. Its greatest value has been in calculating the pH value when both $pCO_2$ and $[BHCO_3]$ are known, but with the present colorimetric methods of determining pH in blood plasma or serum, it is of equal importance in calculating the $CO_2$ tension actually existing in the blood.

The value of $pK_1$ first determined by Hasselbalch (1916–17), has been redetermined by a number of workers, especially by Parsons (1917), Donnegan and Parsons (1919), Warburg (1922), and by Cullen (1917). All writers are agreed that when Hasselbalch put Henderson's equation in logarithmic form, his use of carbonic acid in terms of normality instead of molality was contrary to the usual convention. The pK′ values used here are calculated from the equation

$$pH = pK' + \log \frac{[BHCO_3]}{[H_2CO_3]}$$

where brackets indicate molar concentration or volumes per cent of $CO_2$. The symbol pK′ is used here as in previous reports to differentiate this value from the $pK_1$ of Hasselbalch. There are discrepancies between the values reported by different workers.

Parsons pointed out that the pH determined electrometrically in blood was in reality the pH of serum. Warburg emphasized the fact that the $pK_1$ of Hasselbalch was a composite constant for the heterogenous system (serum and cells) that included factors for cell volume, $O_2$ saturation, etc.

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Warburg also discussed the influence of the presence of hemoglobin and the attendant oxygen error on the accuracy of the electrometric pH determination.

Most of the values for pK′ have been made on ox, horse, or on human blood. Although theoretically the changes in protein and salt concentration between these bloods and abnormal human blood should not be sufficient to change the constant, any change would be so important in pathological physiology and in clinical studies that direct determinations are desirable.

For these reasons, it was considered desirable to redetermine, with the precision of technique now available, the pK′ in human sera from a series of pathological conditions. Serum, not whole blood, was used, because, as pointed out above, it is a simpler system since it can be studied with greater accuracy than blood in regard not only to pH but also to saturation with $CO_2$ and finally because its pH is what is meant by the pH of the blood. Finally, serum was used rather than plasma because it was desired to avoid disturbance of the electrolyte distribution between cells and serum by the oxalate.

The relations between serum values and whole blood values for pK′ may be calculated most accurately by use of the relations established by Warburg (1922), by Peters, Bulger, and Eisenman, and by Van Slyke, Wu, and McLean, which are based upon the principle that the difference in the $[BHCO_3]$ of serum and of blood is a function of cell volume.

Warburg[1] worked out a factor $\log \psi \, CO_2^1$ relating the pK′ of serum and blood. Peters, Bulger, and Eisenman (1924) determined this factor, $\Delta$ pK′, on a large series of human bloods and plasma by the most direct method, namely that of determining the total $[CO_2]$ of the blood and of the plasma after saturation at known $CO_2$ tension. Van Slyke, Wu, and McLean (1923) determined the same value ($pK_B' - pK_s'$) for the blood of one horse.

Both Peters, Bulger, and Eisenman's, and Van Slyke's values are in close agreement and are higher than Warburg's.

### *Standardization of pH Values.*

One of the most puzzling questions in regard to the hydrion concentration of blood or serum is that of the standard of reference for the determination. The arguments for and against the standardization of hydrion concentration to the newer activity value have been summarized by Clark (1920) who decides that because of the present uncertainty of the values for the activity coefficient and because most of the biological work has been standardized to the conductivity values, it is better to continue to use these values. He proposes then a series of values for the N/10 calomel

[1] Warburg (1922), p. 219.

electrode which is to be used as the provisional standard. More recently Sørensen with Linderstrøm-Lang (1924) has supported Clark in this dictum, so that we may accept as final the decision to refer all biological hydrion values to the conductivity basis and to continue to call these values pH values to distinguish them from log of activity values of G. N. Lewis and Bjerrum, which Sørensen and Linderstrøm-Lang designate as $pa_H$.

In attempting to establish the relationship between the pH at room temperature and that at body temperature, we had previously adopted (Cullen 1922, *a*) the use of N/10 HCl as the most reproducible system. We preferred a direct standardization with a reproducible solution to the indirect standardization with the tenth normal calomel electrode, for our experience, and that of many other workers, has been that the tenth normal calomel electrode is liable to variation. When prepared from especially pure material and when continually checked with new electrodes as in Clark's and Sørensen's laboratories it is undoubtedly satisfactory, but in laboratories where the interest and experience in standardizing is not so great, it is apt to give an entirely unwarranted sense of reliability. The N/10 calomel electrode may be constant at its theoretical value for some time and then suddenly become variable (for example see Fales and Mudge (1920)). On the other hand a standard HCl solution is reproducible anywhere, and is stable, and since it is the ultimate standard used in determining the value of the standard electrode, it seems safer, as well as more logical, to use it as the working standard.

Moreover, when in the future the relation of temperature change to pH and to activity change becomes more definitely established it will be a simple matter to correct all values which are based directly upon reproducible solutions.

Cullen (1922, *a*) adopted the activity values for N/10 HCl given by Noyes and Ellis (1917) because we found that these values, when used without correction for diffusion potential, gave, within 0.01 pH, Sørensen's values at 20° for his standard phosphate solutions. By mistake the values for N/10 HCl were reported as $pH_{38°} = 1.090$ and $pH_{20°} = 1.085$. The value of 1.080 which is correct for 38° is now used at all temperatures from 15–40°. This gives even more exact agreement (within 0.005 pH) with Sørensen's phosphate solution at 20° (see also Hastings and

Sendroy (1924)) and has therefore been used as an arbitrary value, easily reproducible, which gives those values at 20° for the standard phosphate which have been used so widely in biological work. We have preferred to use the same value for 38° and 20° because of the uncertainty of the actual temperature correction.

However, now that both Clark and Sørensen are agreed upon the use of the tenth normal calomel electrode at all temperatures, it is desirable to know what relationship our system has to that electrode.

We have no direct determination of our N/10 acid against the tenth normal calomel electrode, but we have indirect measures. Since the solution 0.01 N HCl + 0.09 N KCl has been so widely studied, since it eliminates the diffusion potential, and since it is the solution used by Sørensen and Linderstrøm-Lang (1924) in their recent work, it would seem to be the desirable standard of

TABLE I.

| Temperature. | N/10 HCl | 0.01 N HCl 0.09 N KCl | Sørensen's M/15 $PO_4$, pH = 7.4. |
|---|---|---|---|
| | Assigned pH. | Observed pH. | Observed pH. |
| °C. | | | |
| 20 | 1.080 | 2.05 | 7.40 |
| 38 | 1.080 | 2.04 | 7.37 |

reference for pH work. The pH of such a solution in our system is shown in Table I.

The phosphate difference of 0.03 for 18° of temperature has been repeated many times and holds for the entire Sørensen phosphate series, (confirmed recently by Hastings and Sendroy (1924)) but the values for the solution, 0.01 N HCl + 0.09 N KCl, represent the average of two determinations only, and are therefore provisional but serve to show that at 20° we are probably in agreement with Sørensen's value to 0.01 pH. The temperature difference between 38° and 20° will probably be more, rather than less, than 0.01 pH, but left at its assigned value it happens to give the rounded value, see below, of 6.10 for the pK′ of human serum, which is also a value that has been used extensively in blood studies.

In view of the lessened diffusion correction, it will probably be

better to use the solution (0.01 N HCl in 0.09 N KCl) in the future as the reference standard with the value 2.04 at 38°.

*Technique.*

The blood was withdrawn without stasis from an arm vein into tubes under oil. The serum was obtained in either of two ways, depending upon the time required to draw the blood. If possible the blood was stirred rapidly but gently with a glass-footed rod always in the same direction for about 5 minutes by which time the fibrin is all wrapped around the rod. This technique which was introduced by Dr. H. C. Gram is the only one by which we have been able to defibrinate dog blood consistently without hemolysis.

If there was any evidence of clotting, the clotting was allowed to continue until completion and then the fibrin was loosened from the glass with a platinum wire.

In either case the tube containing the blood was stoppered, as described in the technique for colorimetric pH determination, and centrifuged. The serum was then removed to a sampling tube or to a test-tube under oil. No serum which showed hemolysis was used.

*Equilibration with $CO_2$ at Known Tensions.*

The equilibration was carried out by the second saturation method previously described (Austin *et al.* (1922)). The buffer curve of serum is so flat that two successive saturations are sufficient. The $CO_2$ concentration in the tonometer was attained by measuring the $CO_2$ in a burette over mercury. An improvement in the measuring system over that previously described consists in the simple device of placing a small tube alongside the burette connected to it at the bottom and open to air above (see Fig. 1, *A*). One can then sight across the mercury surface in the burette to the mercury surface in the small parallel tube which is at atmospheric pressure and be sure that the gas inside the burette is also at atmospheric pressure. (The final reading is made with a lower cock (No. 2) closed, and the slight difference in meniscus level due to difference in capillarity of the two tubes is the same at every reading of the burette and introduces an entirely negligible error in the volume measured.)

In manipulating this arrangement, one learns to work both cocks 1 and 2 at the same time, the leveling bulb *B* being in place above the burette and serving as a reservoir. The reading with No. 1 closed is repeated several times to insure accuracy.

The transfer between first and second saturation was carried out, as described before, rapidly and with the tonometer jacketed in warm wet towels. After the second saturation, we introduced an improvement in the technique of transferring the serum to the mercury sampling tube. We used a large water bath, electrically

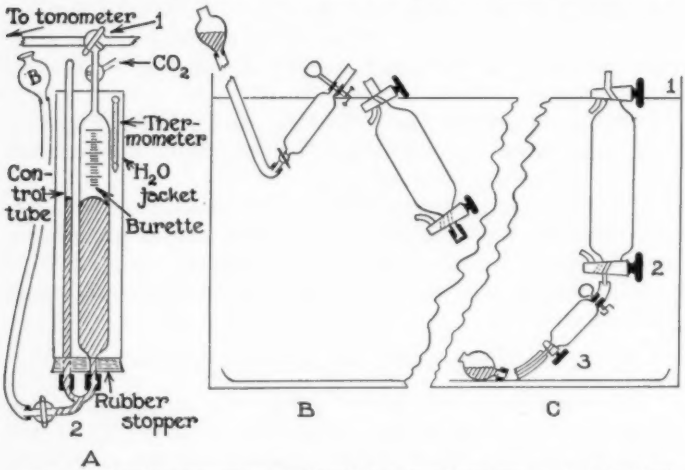


FIG. 1. Showing apparatus employed in saturating serum. A. Modified gas burette; B, technique for transferring saturated serum from tonometer to container without loss of $CO_2$ or change in temperature.

heated and controlled, in which a rotating shaft carried the tonometers. The tonometer after the second saturation was released from its support and brought to the position shown in *B*, Fig. 1. The rubber cap which protected the outlet tube from water was removed and the sampling tube connected. A trace of mineral oil on the edge of the rubber tube facilitates this since the tube must fit so snugly that there is no danger of its being pulled off. We find the transparent, acid-cured, rubber tubing (A. H. Thomas Co., No. 8840) best for this purpose as well as for drawing blood.

Next, always under water, the tonometer and sampling tube

are inverted to position *C*. When cock 3 is open, mercury is run through cock 2 to the outlet to remove all air. Nos. 2 and 3 are now closed and the levelling bulb lowered to below the sampling tube. Then Nos. 1 and 2 are opened and the sample drawn into the sampling tube by manipulation of No. 3. When the surface of the sample in the tonometer reaches No. 2, No. 3 is closed, the pinch clamp, which had been resting over the glass tube, snapped in place, the leveling bulb raised from the bath, and No. 3 opened. The whole is now removed from the bath, the tonometer disconnected, and the sampling bulb placed in its support with the serum under pressure. Thus contraction during cooling can cause no extraction of gas.

It is most convenient for two persons to assist each other with this technique. One holds the tonometer and leveling bulb, the other holds the sampling tube in one hand while he makes connections and manipulates the cocks with the other.

For removal of samples, cock 3 is closed, the pinch clamp removed, a drop or two wasted from the rubber tube, then the pipette inserted into the rubber tube. The pipette is filled by pressure controlled by cock 3. For pH determination a small 5 cc. burette is used, containing about a centimeter layer of mineral oil. The tip of the burette is filled with oil before insertion into the rubber tip of the sampling tube.

*Total $CO_2$ Determinations.*—The total $CO_2$ determinations were made with Van Slyke and Neill's (1924) constant volume technique, using 1 cc. samples. Duplicate determinations almost always were in agreement within less than 0.1 mm.

*Conductivity of the Serum.*—The conductivity was determined by the ionometer method of Christiansen and the results expressed in NaCl equivalents, corrected for protein (Gram and Cullen (1923)) which was determined refractometrically.

*Hemoglobin.*—The hemoglobin was determined colorimetrically in an Autenrieth colorimeter, standardized by $O_2$ determinations by Van Slyke and Stadie's (1921) technique. We are indebted to Dr. H. C. Gram for these and the conductivity determinations.

### *Calculations.*

*$CO_2$ Tension.*—Bohr and Bock's values for $CO_2$ solubility in serum were used throughout (*i.e.* 97.5 per cent of water solubility).

*pH Determinations.*

*Electrometric pH Determinations.*—These were made as described before by Cullen (1922, *a*) by a combination of Höber's gas mixture method and Hasselbalch's refill technique. A small 2 cc. Clark electrode, modified for temperature control (Cullen (1922, *b*)) was used. When the pH determinations were made at different temperatures on the same solution the $CO_2 + H_2$ mixture was calculated to give the same $[H_2CO_3]$ content in the solution.

Our procedure was to use freshly prepared platinum electrodes for each serum and to determine the "e" $\left(\text{pH} = \frac{\text{E.M.F.} - \text{"e"}}{0.0001984\ \text{T}}\right)$ of the entire system (including calomel cell and platinum electrode) against standardized phosphate solution, before and after each determination.

*Colorimetric pH.*—These determinations were made on 1 or 0.5 cc. samples with the technique described before (Cullen 1922, *a*) except that the phenol red was measured with a pipette graduated to 0.01 cc. The phenol red was adjusted to such strength (about 0.04 per cent) that 0.01 cc. was used per 1 cc. of standard solution. 100 cc. of 0.9 per cent NaCl solution containing 1.05 cc. phenol red solution was adjusted to pH about 7.5. Colorimetric determinations are indicated by brackets, thus, [pH], to distinguish from electrometric determinations.

*Calculations.*

The formula used for calculating gas mixtures for saturation and $[H_2CO_3]$ concentration are the same as those given in an earlier paper (Austin *et al.* (1922)). For the $CO_2$ saturation mixtures, Formula V,[2] and for mM $[H_2CO_3]$ Formula I,[3] were used.

As a matter of fact we used the formula in the mM form, estimating $\text{mM}_{\text{solution}}$ from desired $\text{mM}_A$. $\text{mM}_s = \alpha' \text{mM}_A$.

$$\text{cc. } CO_2 = \frac{\text{mM}_A \times (V_T - V_S)}{16.04} \times \frac{T \text{ burette}}{B - W_B}$$

Where $\text{mM}_A$ = concentration of $CO_2$ in the gas phase of the tonometer in millimols per liter.

[2] Austin *et al.* (1922), p. 140.

[3] Austin *et al.* (1922), p. 144.

$V_T$ = volume of tonometer in cc.
$V_S$ = volume of solution in tonometer in cc.
$T$ burette = absolute temperature $C$ at which $CO_2$ is measured.
$B$ = barometric pressure in mm.
$W_B$ = vapor pressure of water in mm. at temperature $T$ burette.
cc. $CO_2$ = cc. of $CO_2$ run from the burette into the tonometer.

In Table II the $pCO_2$ values are given for convenience but are calculated only to 0.1 mm.

For calculation of the $CO_2 + H_2$ mixture for the hydrogen electrode, where the mixture in the electrode is equilibrated at atmospheric pressure

$$\text{cc. } CO_2 = \frac{pCO_2 \cdot V}{(B - W)}$$

when both $CO_2$ and $H_2$ are at the same temperature and where $pCO_2$ = $CO_2$ tension in mm., $V$ = volume of the tonometer in cc., $B$ = barometric pressure in mm., and $W$ = vapor pressure of water at the temperature of the electrode.

*Correction to Constant $[H_2CO_3]$ and Constant pH.*—The $[H_2CO_3]$ at the two temperatures was planned to be almost the same. Since the correction to constant $[H_2CO_3]$ was therefore small, it was considered sufficient to assume that the slope $\frac{d[BHCO_3]}{d[H_2CO_3]}$ is a straight line. The values from human separated serum of Doisy, Briggs, Eaton, and Chambers (1922) seem best for this purpose.

From the protocols for separated serum at the end of their article one can calculate the following value for $\frac{d[BHCO_3]}{d[H_2CO_3]}$ between 40 and 70 mm. $pCO_2$.

| | Ratio. |
|---|---|
| For J. M. | 0.92 |
| E. A. D. | 1.22 |
| W. H. C. | 1.36 |
| Average | 1.17 |

We have taken as an approximate ratio 1.2.

The correction to constant pH is greater than to constant $[H_2CO_3]$ because the pH values are further apart. Since we had no data for the buffer slope of human serum at 20°, we corrected the 38° $[BHCO_3]$ to the pH at 20°, using the fact that $\frac{d[BHCO_3]}{dpH}$

TABLE II.

*Summary of Data*

| No. | Condition. | Determinations on serum. | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | As drawn. | | | Equilibrated. | | | | | |
| | | Blood hemoglobin. | Protein. | Conductivity NaCl equivalent. | Temperature of saturation. | pCO | Total $[CO_2]$. | $pH_S$* | $pH_S$* | Color reading $[pH]_S$.* |
| | | *per cent* | *per cent* | *mm.* | *°C.* | *mm.* | *mM* | | | |
| 1 | Nephrits. | | 6.8 | 0.134 | | | 25.45 | 7.280 | | [7.53]* |
| 2 | Pulmonary tuberculosis. | | 7.8 | 0.129 | 38 | 40.6 | 35.75 | 7.507 | | |
| 3 | Primary pernicious anemia. | 56 | 7.1 | 0.138 | 38 | 34.0 | 25.32 | 7.448 | | [7.78] |
| 3 | Same 5 days later. | 56 | 6.5 | 0.140 | 38 | 50.4 | 28.75 | 7.334 | | [7.67] |
| 4 | Achylia gastrica. | 95† | 7.9 | 0.134 | 38 | 45.0 | 29.30 | 7.395 | | [7.75] |
| | | | | | 20 | 28.6 | 31.02 | | 7.490 | [7.55] |
| 5 | Cardial decomposition lues. | 91 | 9.0 | 0.138 | 20 | 28.6 | 29.74 | | 7.456 | [7.53] |
| 6 | Anemia simplex. | 40 | 8.4 | 0.137 | 38 | 40.7 | 25.44 | 7.382 | | [7.72] |
| | | | | | 20 | 25.4 | 27.19 | | 7.498 | [7.53] |
| 7 | Hyperthyroid. | 95 | 8.3 | 0.134 | 38 | 40.0 | 28.97 | 7.441 | 7.660 | [7.70] |
| 8 | Primary pernicious anemia. | 29 | 6.6 | 0.134 | 38 | 60.0 | 26.40 | 7.214 | | [7.46] |
| 9 | Carcinoma, liver. | 82 | 7.8 | 0.126 | 38 | 59.6‡ | 29.00 | 7.281 | | [7.54] |
| | | | | | 20 | 37.6 | 31.04 | | 7.357 | |
| 10 | Diabetes. | 99 | 9.6 | | 38 | 59.6 | 29.99 | 7.271 | | [7.59] |
| | | | | | 20 | 37.8 | 32.02 | | 7.390 | [7.43] |
| 11 | Carcinoma, liver. | 70 | 7.4 | 0.118 | 20 | 37.7 | 29.45 | | 7.371 | [7.36] |
| 12 | Diabetes. | 95† | 7.8 | | 38 | 48.4 | 28.37 | 7.343 | | [7.63] |
| | " (plasma). | | | | 38 | 48.6 | 29.16 | 7.343 | | [7.58] |
| 13 | Pneumonia infection. | 90 | 6.1 | 0.120 | 38 | 40.4 | 31.52 | 7.453 | | [7.72] |
| 14 | " | 105† | 8.3¶ | 0.133 | 38 | 60.2 | 31.55 | 7.263 | | [7.62] |
| 15 | Rheumatic fever. | 100† | 8.3¶ | 0.129 | 38 | 40.0 | 25.98 | 7.364 | | |
| 16 | Mixed from two patients. | 105 | 8.5 | 0.135 | 38 | 40.0 | 28.45 | 7.422 | | |
| 17 | Epidemic encephalitis. | 97 | 9.0 | 0.134 | | | | 7.360 | | [7.69] |
| Dog 20 | Normal. | | | | 20 | 24.2 | 23.10 | 7.102 | 7.435 | [7.44] |
| " 21 | " | | | | 38 | 44.4 | 25.60 | 7.317 | 7.500 | [7.62] |
| " 22 | " | | 7.0 | 0.148 | 38 | 42.2 | 21.38 | 7.276 | | [7.43] |
| " 23 | " | | 6.4 | 0.143 | 38 | 39.7 | 23.70 | 7.371 | 7.550 | [7.65] |
| " 23 | " | | | | 20 | 21.3 | 23.92 | 7.350 | 7.505 | |
| Average (human) | | | | | | | | | | |
| Average total | | | | | | | | | | |

* Brackets indicate reading on 22 × diluted serum.

† Estimated from measuring centrifuged cell volume.

‡ $CO_2$ added in two portions.

§ Plasma.

¶ Very high fibrin.

TABLE II.
*Summary of Data.*

| Color reading $[pH]_{38°}$ | $[BHCO_3]$ | $[H_2CO_3]$ | log R | Calculated. $pK'_{38°}$ | $pK_{38°}$ | $[pH]_{38°} - pH_{38°}$ C | $[pH]_{38°} - pH_{38°}$ |
|---|---|---|---|---|---|---|---|
| | *mM* | *mM* | | | | | |
| [7.53]* | | | | | | 0.25 | |
| | 34.46 | 1.291 | 1.426 | 6.081 | | | |
| [7.78] | 24.24 | 1.078 | 1.356 | 6.092 | | 0.31 | |
| [7.67] | 27.15 | 1.600 | 1.230 | 6.104 | | 0.34 | |
| [7.75] | 27.87 | 1.430 | 1.290 | 6.095 | | 0.35 | |
| [7.55] | 29.58 | 1.437 | 1.314 | | 6.177 | | 0.06 |
| [7.53] | 28.30 | 1.440 | 1.293 | | 6.163 | | 0.07 |
| [7.72] | 24.15 | 1.293 | 1.271 | 6.110 | | 0.34 | |
| [7.53] | 25.91 | 1.277 | 1.309 | | 6.190 | | 0.03 |
| [7.70] | 27.70 | 1.270 | 1.339 | 6.102 | | 0.26 | 0.04 |
| [7.46] | 24.49 | 1.905 | 1.109 | 6.105 | | 0.25 | |
| [7.54] | 27.11 | 1.891 | 1.157 | 6.123 | | 0.26 | |
| | 29.15 | 1.891 | 1.188 | | 6.169 | | |
| [7.59] | 28.10 | 1.894 | 1.172 | 6.099 | | 0.32 | |
| [7.43] | 30.12 | 1.900 | 1.200 | | 6.190 | | 0.04 |
| [7.36] | 27.56 | 1.893 | 1.163 | | 6.208 | | 0.01 |
| [7.63] | 26.83 | 1.536 | 1.243 | 6.100 | | 0.29 | |
| [7.58] | 27.62 | 1.541 | 1.253 | 6.090 | | 0.24§ | |
| [7.72] | 30.24 | 1.283 | 1.372 | 6.081 | | 0.27 | |
| [7.62] | 29.64 | 1.910 | 1.191 | 6.072 | | 0.35 | |
| | 24.71 | 1.270 | 1.289 | 6.075 | | | |
| | 27.18 | 1.270 | 1.330 | 6.092 | | | |
| [7.69] | | | | | | 0.33 | |
| [7.44] | 21.89 | 1.214 | 1.256 | | 6.180 | 0.34 | 0.01 |
| [7.62] | 24.19 | 1.408 | 1.234 | 6.093 | | 0.30 | 0.12 |
| [7.43] | 20.05 | 1.328 | 1.178 | 6.098 | | 0.28 | |
| [7.65] | 22.44 | 1.262 | 1.250 | 6.121 | | 0.28 | 0.10 |
| | 22.85 | 1.072 | 1.329 | | 6.176 | | |
| ..... | | | | 6.095 | 6.183 | 0.30 | 0.04 |
| ..... | | | | 6.096 | 6.182 | | |

is approximately linear. We used for the slope the value 6.7 mM which we obtained by averaging the value from the three sera cited above with those from the two plasmas of Peters, Bulger, and Eisenman (1924). The five values give an average $\frac{d[BHCO_3]}{dpH}$ serum 38° = 6.7 mM per 1.0 pH unit.

TABLE III.

| No. | Name. | Age. | Sex. | Race. | Diagnosis. |
|---|---|---|---|---|---|
| 2 | M. L. | 63 | Male. | Russian Jew. | Pulmonary tuberculosis (chronic fibroid). |
| 3 | C. K. | 60 | Female. | American. | Pernicious anemia. |
| 4 | M. B. | 38 | " | Austrian. | Intestinal adhesions gastric anacidity. |
| 5 | E. V. | 43 | Male. | Colored. | Chronic myocardial disease with anasarca. Tertiary lues. |
| 6 | T. R. | 40 | Female. | Russian Jew. | (Hemolysis?) pernicious anemia? Secondary anemia. |
| 7 | L. W. | 17 | " | Jewish. | Hyperthyroidism. |
| 8 | E. J. C. | 54 | Male. | American. | Pernicious anemia. |
| 9 | F. C. | 58 | " | " | Carcinoma, liver. |
| 10 | A. C. | 42 | Female. | " | Diabetes mellitus. |
| 11 | E. C. | 67 | " | English. | Carcinoma, liver. Diabetes mellitus. |
| 12 | A. G. | 32 | Male. | Russian Jew. | Diabetes mellitus (mild). |
| 13 | E. G. | 27 | " | American. | Lobar pneumonia. Pleurisy with effusion. |
| 14 | M. A. | 52 | Female. | Colored. | Lobar pneumonia. |
| 15 | S. | 19 | Male. | American. | Rheumatic fever. |
| 17 | M. K. | 16 | " | " | Epidemic encephalitis dyspituitarism. Diabetes inspidus. |

## DISCUSSION OF RESULTS.

Table II presents a summary of the analyses and calculations on both human and dog sera at both 20° and 38°. The discussion of the results follows. The clinical data in regard to the patients studied are given in Table III.

*pK′ at 38°.*—Fifteen determinations on sera from thirteen individuals are included in this series. The values range from

6.123 to 6.072 with an average of 6.095. The average deviation from this value is 0.010. There are three values which, perhaps, because of their large deviation from this mean, should be excluded. These are Nos. 9, 14, and 15. (In 9 there was a slight difficulty in technique, in 14 and 15 there were unusually high fibrin clots upon defibrination.) If we exclude these three determinations and that of the plasma of No. 12, the average becomes 6.097 with an average deviation of 0.007. The three determinations do not, then, alter the average.

The three values on normal dog sera give an average value of 6.104.

If the total eighteen determinations are considered as a whole the average value is 6.096. It is evident that this value 6.096 represents the pK′ of serum at 38° and apparent that this value is not greatly disturbed by various disease conditions. This value rounded off to 6.10 agrees with the value at present most generally used.

*pK′ at 20°.*—pK values at 20° were obtained on six sera. The average for these six human sera is 6.183. If we include the two normal dog sera, we have practically no change, *i.e.* an average of 6.182 for the series with an average deviation of 0.011.

The average deviation 0.007 and 0.011 at the two temperatures represents about the expected accuracy of the method, *i.e.* about 0.01 pH. This is discussed fully in the paper by Austin *et al.* (1922).

$pK'_{20°} - pK'_{38°}$.—The value for the average temperature difference becomes 6.184 − 6.096 = 0.088 or 0.09. This gives a temperature coefficient of 0.005 pK′ per 1°C.

*Influence of pH, Protein, and Salt on pK′ Variation.*

In view of the change in base bound by protein at different pH, it is important to determine if this influences the pK′ of serum. In Fig. 2, pK′ values are plotted against pH values. It is evident that for our series there is no relation between pK′ and pH and we may conclude therefore that for serum over the possible physiological range (pH = 7.0 to 7.9) the value 6.10 holds.

In Fig. 3, the values are charted against protein concentration. Over the rather extreme concentration of 6 per cent to 9.6 per cent met with in this series, there is no apparent relationship.

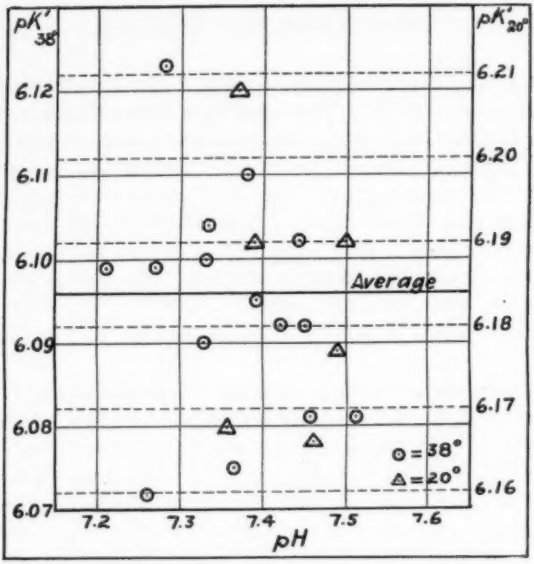


FIG. 2. Relation of pK′ at 38° and 20° to pH at 38°.

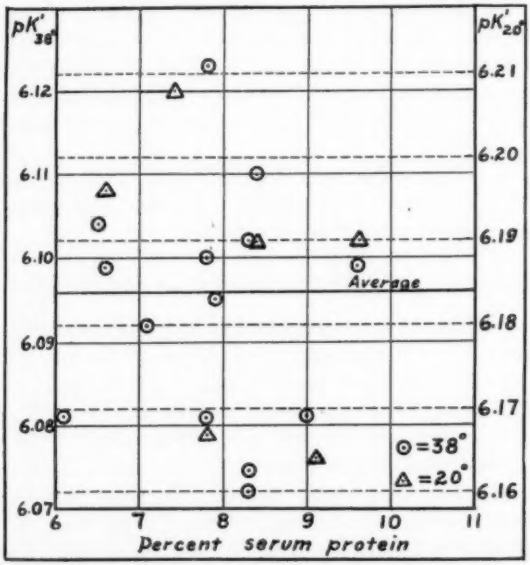


FIG. 3. Relation of pK′ at 38° and 20° to concentration of serum protein.

The total effective salt concentration is best represented by the equivalent conductivity. Using this and expressing the conductivity corrected for protein in terms of equivalent NaCl normality, *i.e.* normality of NaCl which would give the observed conductivity, we find the conditions expressed graphically by Fig. 4.

There is not enough variation here to establish any systematic

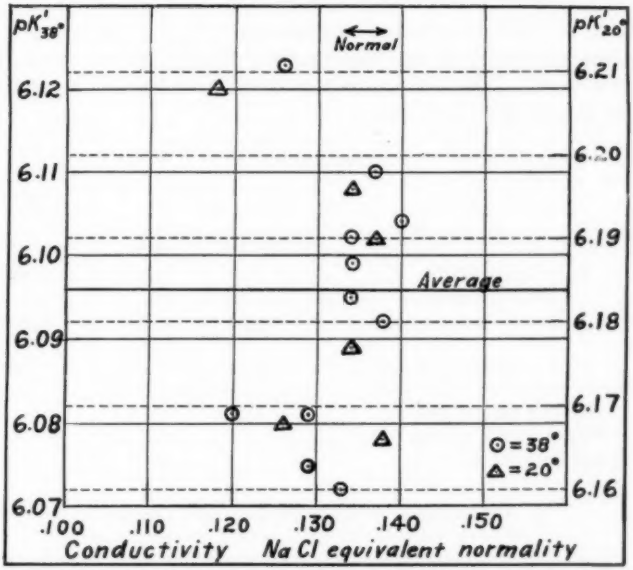


FIG. 4. Relation of pK′ at 38° and 20° to electrolyte concentration in serum.

grouping. It is apparent that the sera with abnormally low conductivity all show comparatively large pK′ variation but include both high and low values. There is also a suggestion that the six lowest pK′ values are grouped about a lower conductivity value. This is contrary to the experience with pure bicarbonate solutions where increase in salt concentration decreases pK′. The series is too small moreover to do more than indicate that there is no marked systematic change with conductivity change.

*pK′ of Plasma.*

There is no reason to expect that the slight change in electrolyte concentration introduced by 0.3 per cent oxalate (=0.02 M) will appreciably affect the results for serum. The value calculated by Van Slyke, Wu, and McLean (1923) from Cullen's (1922, *a*) data gives the same value 6.11 for both horse plasma and serum.

Also in Table II, No. 12, are found the results of an experiment in which blood was drawn simultaneously, by means of two tubes and a Y tube, for plasma and serum. The original total $CO_2$ values for the serum and plasma were 28.37 mM and 29.16 mM, respectively. The pK′ values reported here may then be considered as accurate for plasma.

TABLE IV.

| | pK′ | | $\frac{dpK'}{dt}$ |
|---|---|---|---|
| | 38°C. | 18°C. | |
| $NaHCO_3 + NaCl$* [Na] = 0.16 N. | 6.110 | 6.210 | 0.005 |
| Serum: ox and horse† | 6.096 | 6.235 | 0.007 |
| | | 20° | |
| " human and dog‡ | 6.096 | 6.182 | 0.005 |

* Warburg (23), p. 242.
† Warburg (23), p. 209.
‡ Author's value.

*Comparison of pK′ with Previous Values.*

The value 6.10 compares well with the value of 6.11 calculated by Van Slyke, Wu, and McLean (1923) for horse serum and plasma from Cullen's (1922, *a*) data (reported as 6.12, now corrected to our present pH standard).

Warburg's (1922) determinations for 0.16 N bicarbonate in saline and for horse and ox serum all reduced by 0.048 from Bjerrum to Sørensen's pH values compare with ours as shown in Table IV.

The agreement at 38° is satisfactory. The change with temperature for our serum and Warburg's salts is also in agreement but there is a discrepancy between Warburg's temperature variation for serum and ours.

*Change in [$BHCO_3$]-Combining Capacity with Change in Temperature.*

It is known that the blood and serum bind increasing amounts of $CO_2$ with decrease in temperature (Warburg (1922), Stadie and Martin (1924)). The above series includes five human sera and one dog serum which were studied at two temperatures and which therefore afford opportunity to evaluate the value $\frac{d[BHCO_3]}{dt}$

TABLE V.

*Changes in [$BHCO_3$] of Separated Serum with Change in Temperature at (a) Constant pH and (b) at Constant [$H_2CO_3$].*

| Serum. | Temperature. | [$BHCO_3$] | [$H_2CO_3$] | [$BHCO_3$] − 38° corrected to [$H_2CO_3$] at 20°. | pH | [$BHCO_3$] − 38° corrected to pH at 20°. | $\Delta[BHCO_3]_{20°-38°}$ at constant | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | [$H_2CO_3$] | pH |
| | °C. | mM | mM | mM | | mM | mM | mM |
| 4 | 38 | 27.87 | 1.437 | 27.88 | 7.395 | 27.23 | 1.70 | 2.35 |
| | 20 | 29.58 | 1.430 | | 7.490 | | | |
| 6 | 38 | 24.15 | 1.293 | 24.13 | 7.382 | 23.37 | 1.78 | 2.54 |
| | 20 | 25.91 | 1.277 | | 7.498 | | | |
| 9 | 38 | 27.11 | 1.891 | 27.11 | 7.281 | 26.60 | 2.04 | 2.55 |
| | 20 | 29.15 | 1.891 | | 7.357 | | | |
| 10 | 38 | 28.10 | 1.894 | 28.11 | 7.271 | 27.30 | 2.01 | 2.82 |
| | 20 | 30.12 | 1.900 | | 7.390 | | | |
| Dog 23 | 38 | 22.44 | 1.262 | 22.21 | 7.371 | 21.54 | 0.64 | 1.31 |
| | 20 | 22.85 | 1.072 | | 7.505 | | | |
| Average for human.. ..... | | | | | | | 1.88 | 2.56 |

under various conditions. This has been done for two conditions, constant [$H_2CO_3$] and constant pH. Constant [$H_2CO_3$] may be considered as constant effective $CO_2$ tension, *i.e.* the $CO_2$ tension which gives at the various temperatures the same concentration of $H_2CO_3$.

The condition of constant pH when used for changing temperature introduces the questions of pH standardization and of the relation between the [$H^+$], the [$OH^-$], and the neutral point (see Austin and Cullen (1) for further discussion of this point.)

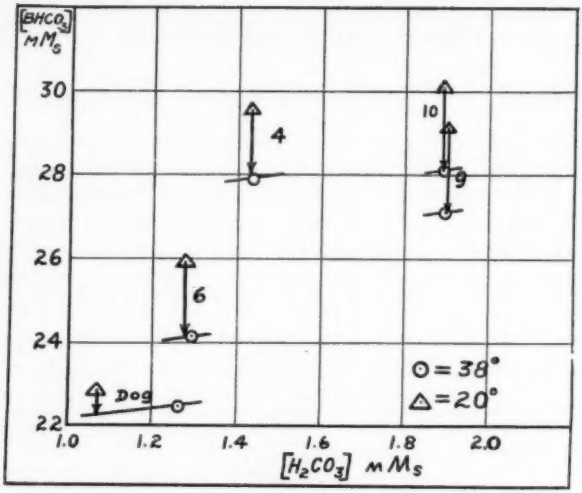


FIG. 5. Showing change in $[BHCO_3]_{38-20°}$ at constant $[H_2CO_3]$ in separated serum. Δ = observed values at 20°, ○ = observed values at 38°. Corrections to constant $[H_2CO_3]$ indicated by sloping lines.

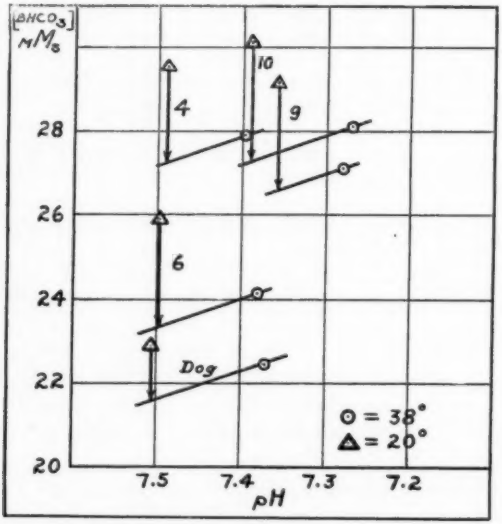


FIG. 6. Showing change in $[BHCO_3]_{38-20°}$ at constant pH in separated serum. Δ = observed values at 20°, ○ = observed values at 38°. Corrections to constant pH indicated by sloping lines.

The changes in $[BHCO_3]$ calculated for both constant $[H_2CO_3]$ and constant pH are given in Table V. The factors used in calculating this table are given above under Calculations.

The $pCO_2$ tensions were planned to give close agreement in $[H_2CO_3]$ at the two temperatures so that the error in correcting to the same $[H_2CO_3]$ would be negligible.

The corrections and the resulting $d[BHCO_3]$ values are recorded graphically in Figs. 5 and 6.

The average value for the five human separated sera for $d[BHCO_3]$ at constant $[H_2CO_3]$ is

$$\left(\frac{d[BHCO_3]}{dt^\circ}\right)_{[H_2CO_3]} = -\frac{1.88}{18} = -0.10 \text{ mM per } 1^\circ\text{C.}$$

The average value for $d[BHCO_3]$ at constant pH for 18° is

$$\left(\frac{d[BHCO_3]}{dt^\circ}\right)_{pH} = -\frac{2.56}{18^\circ} = -0.14 \text{ mM per } 1^\circ\text{C.}$$

These data are in close agreement with those given by Austin and Cullen (1925, Table II) where in addition the corresponding changes for whole blood are calculated.

Such data offer no basis for distinguishing between the relative importance *in vivo* of $[CO_2]$, $[H_2CO_3]$, $pCO_2$, or pH under conditions of changing temperature in regulating the respiratory center and the acid-base balance in the blood. Depending upon which factor remains constant *in vivo* the variation in serum pH per degree change in temperature may vary from 0.00 to −0.02. Furthermore, it must be remembered that these calculatons are based upon the assumption of constant available total base in the blood for combination with protein and $HCO_3^-$, a condition which probably often does not hold in temperature changes *in vivo*.

*The C of the Colorimetric Determination of pH of Serum.*

The determination of the pH of normal plasma, colorimetrically, has proved accurate and satisfactory when carried out as previously reported (Cullen (1922, *a*)) on dilute plasma. The plasma read at room temperature, t°, is corrected to 38°C. by the equation

$$pH_{38^\circ} = [pH]_{20^\circ} - C$$

where $[pH]_{20°} = [pH]_{t°} + 0.01\ (t° - 20°)$ and [pH] indicates *colorimetric* pH reading. $C$, for human plasma, has an average value of 0.23.

The value of this method has been confirmed by further results in our laboratories and by Marrack and Smith (1924) and by Hastings and Sendroy (1924).

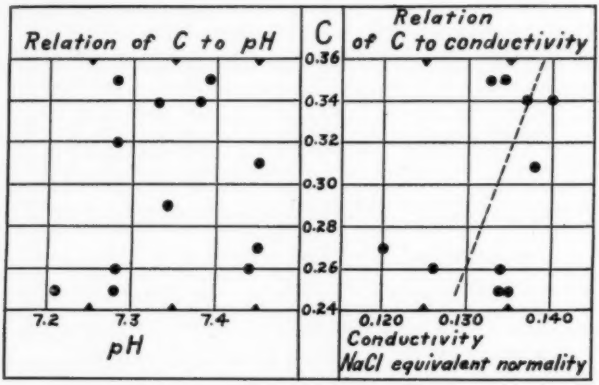


FIG. 7. Relation of $C$ to pH and to conductivity.

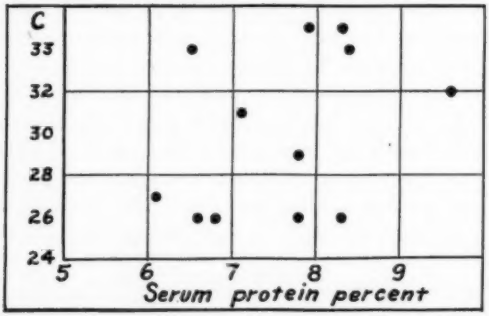


FIG. 8. Relation of colorimetric factor $C$ to serum protein concentration.

The experiments reported above afforded an opportunity to study the value of $C$ for human serum. The values for $C$ are found in Table II. All colorimetric readings are corrected to 20° by the correction factor, 0.01 per 1° used for plasma.

It is apparent that the $C$ for these abnormal sera has not the

same degree of constancy that it has for normal human and horse plasma. In an attempt to find some explanation for this discrepancy the $C$ serum values have been plotted (Figs. 7 and 8) against $pH_{38°}$, against protein content, and against conductivity.

It is at once evident, that the twelve determinations fall into two groups, one with an average of 0.26 and the other with an average of 0.33 but our data offer no explanation of this grouping. From the figures it can be seen that the variation is not related to serum protein or to the pH of the serum itself. This is in agreement with the work of Marrack and Smith (1924), who found that change in protein concentration had no influence on the correction $C$ for plasma.

There is a suggestion in the conductivity plot of Fig. 6 that the lower group has a lower conductivity but this difference is not sharp enough to account for the distinct grouping. To the $C$ values for dog sera given above may be added two more, determined on the same dog before and after tetany, of 0.30 and 0.29. This gives an average for dog sera of 0.30 which is consistent with that previously used. There is reason to believe, however, that the variation of $C$ in the dog is greater than in man. Moreover, the extent to which this correction varies under pathological conditions has not yet been adequately determined.

*The Relation between Colorimetric [pH] and Electrometric pH at 20°.*—In the last column of Table II are given a few determinations of the colorimetric $[pH]_{20°}$ of diluted serum compared with the electrometric $pH_{20°}$ undiluted, the average correction to the colorimetric reading is $-0.04$ pH.

## SUMMARY.

1. The standardization of pH determination at 38° and 20° is discussed.

2. $pK'_{38°}$ determined on fifteen pathological human sera gives an average of 6.095 ($CO_2$ solubility coefficients of Bohr and Bock being used in the calculation).

3. $pK'_{20°}$ for the same sera is 6.183.

4. The values for pK′ for dog sera agree with those for human sera.

5. There is no evidence that the nature of the disease, possible salt content variation, protein variation, or pH itself significantly affect these values.

6. The temperature coefficient for pK′ is −0.005 per 1°C.

7. The temperature coefficient for the $CO_2$-combining capacity of human separated serum at constant $[H_2CO_3]$ and at constant pH are calculated as

$$\left(\frac{d[BHCO_3]}{dt^\circ}\right)_{[H_2CO_3]} = -0.10 \text{ mM per } 1°C.$$

$$\left(\frac{d[BHCO_3]}{dt^\circ}\right)_{pH} = -0.14 \text{ mM per } 1°C.$$

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# CARBON AND NITROGEN TRANSFORMATIONS IN THE DECOMPOSITION OF CELLULOSE BY FILAMENTOUS FUNGI.*

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## INTRODUCTION.

The nature of cellulose decomposition by microorganisms has attracted considerable attention, because of the fact that this process completes the circle of transformation of carbon in nature, especially since carbon is the largest single constituent of plant tissues and cellulose is rather resistant to decomposition. However most of the investigations on the chemistry of cellulose transformation have been limited largely to bacteria. In the case of fungi attention has been paid mainly to establishing the types of organisms capable of breaking down celluloses and utilizing them as the sole source of energy and carbon.

It has been pointed out elsewhere (Waksman and Skinner (10)) that the decomposition of celluloses in well aerated humid soils, especially those of an acid reaction, is carried on largely by fungi. A number of organisms belonging to different groups were found capable of decomposing celluloses more or less rapidly. Here belong species of *Aspergillus*, *Penicillium*, *Trichoderma*, *Fusarium*, *Verticillium*, *Humicola*, etc. The actinomycetes can decompose celluloses only to a limited extent; the rôle of these organisms in the soil seems to consist not so much in the decomposition of celluloses, but of lignins and of the cells synthesized by the soil fungi and other microorganisms, as will be shown elsewhere. Results presented in this paper deal with the transformation of carbon and

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nitrogen in the process of cellulose decomposition by two typical soil fungi, namely a species of *Trichoderma* (*Trichoderma köningi* Oud.) and a species of *Penicillium* (Soil Group III, Waksman (7)). These organisms were isolated from the soil by the authors.

*Methods.*

In the studies reported below cellulose was used in the form of filter paper (Whatman's No. 2). In all cases 1 per cent of paper was added to the culture medium, which was a synthetic solution, or a sand medium or soil. In solution cultures the filter paper was introduced into flat bottom long necked flasks, of 300 cc. capacity, and spread on the surface of the solution so as to give maximum exposure of the paper. In the case of sand and soil cultures very finely ground filter paper was mixed in with the soil or sand; the latter had been thoroughly washed with HCl and water. The necessary amount of minerals was introduced as indicated below. The flasks were then plugged with cotton and fitted with rubber stoppers with two delivery tubes, the ends of which were also plugged. The flasks and connections were sterilized for $\frac{1}{2}$ hour at 15 pounds pressure in the case of solution culture and for $1\frac{1}{2}$ hours in the case of sand or soil cultures. They were then inoculated with a loopful of the spore material of a recently transplanted culture, and connected in the incubator (25–28°C.), to the respiration apparatus for measuring the $CO_2$ evolved (Waksman and Starkey (12)). The flasks were aerated every day for an hour by passing $CO_2$-free air over the medium and then absorbing the $CO_2$ produced by the culture in large test-tubes containing a standard solution of $Ba(OH)_2$ solution. The excess $Ba(OH)_2$ was titrated with oxalic acid using phenolphthalein as an indicator. At the end of each incubation period two or three flasks were taken out and different determinations made on representative samples. When $CaCO_3$ had been used in the medium, the remaining $CO_2$ was expelled, at the end of the incubation period, by adding 5 cc. of 4 per cent $H_2SO_4$ to the cultures in the original flasks to decompose the carbonates; the $CO_2$ thus liberated was absorbed in fresh tubes with $Ba(OH)_2$. The values thus obtained were subtracted from those of uninoculated control flasks treated in the same manner, giving the amount of $CO_2$ freed from the carbonates during the incubation period. By subtracting the $CO_2$ formed from the carbonates

from the total amount of $CO_2$ produced during the incubation period, the amount of $CO_2$ liberated as a result of cellulose decomposition was obtained.

The cellulose was determined by the method of Charpentier (2). This consists in extracting the cellulose with Schweitzer's reagent and precipitating an aliquot portion of the filtered extract with 80 per cent alcohol. The residue is filtered through Gooch crucibles, thoroughly washed with alkali, acid, and water, dried, weighed, ignited, and weighed. The difference between the dry and ignited weight of the crucibles gives the weight of undecomposed cellulose.

The total carbon was determined by the wet combustion method as described by Waksman and Starkey (11). According to this method the organic matter is oxidized with $KMnO_4$ and $H_2SO_4$, the gases are passed through a condenser, and the chlorides are freed by absorption in $AgSO_4$ solution; the $CO_2$ is then absorbed in a series of test-tubes containing standard $Ba(OH)_2$ solution, which is then titrated with standard oxalic acid solution.

The ammonia determinations were made by extracting the soil or sand culture with N/2 KCl and filtering, until a drop of the filtrate gave no further positive test for ammonia. The filtrate thus obtained was distilled with MgO into standard acid. Where the culture medium contained nitrates, in addition to ammonia, the filtrate was first distilled with 5 cc. of NaOH solution (42 per cent by weight). The ammonia was absorbed in standard acid solution and titrated. The liquid was then made up to its original volume, 3 gm. of Devarda alloy was added, and distilled a second time. The second titration gave the amount of nitrates as ammonia. The difference between the nitrogen present in the control and inoculated flasks, as determined by this method, was taken to represent the nitrogen assimilated by the organism. By distilling the replaced ammonia in the KCl extract rather than in the whole material, the objection that MgO would break up the amide linkages of the microbial protoplasm present in the cultures was removed.

Uninoculated controls of the same media were always prepared for each experiment; the inoculated cultures were in duplicates or triplicates, but only the averages are reported in the tables.

*Experiment 1. Decomposition of Cellulose by Trichoderma sp.*

*in Solution Culture.*—A nutrient solution having the following composition was first used:

| | |
|---|---|
| $K_2HPO_4$ | 2.0 gm. |
| $MgSO_4$ | 2.0 " |
| NaCl | 0.2 " |
| $(NH_4)_2SO_4$ | 4.0 " |
| $FeSO_4$ | 0.04 " |
| Distilled water | 1000 cc. |

50 cc. portions of this solution were introduced into a series of flasks each containing 1 gm. of filter paper. The flasks were sterilized; 200 mg. portions of sterile $CaCO_3$ were added to each and cultures inoculated and incubated for different periods. At the end of each incubation period two flasks were removed from the incubator, the contents were filtered, and the residue washed thoroughly.

Aliquot portions of the filtrate were analyzed for ammonia, total nitrogen, and total carbon. The residue was dried, weighed, ground, and used for cellulose determination. Exactly the same procedure was followed in the case of the two control flasks. All the results are calculated on the basis of the total contents of the cultures and only the average of the two flasks is given in the tables.

Table I shows an interesting correlation between the amount of cellulose decomposed, $CO_2$ produced, and nitrogen assimilated by the organism. Increasing periods of incubation gave larger amounts of cellulose decomposed and corresponding increases in the evolution of $CO_2$ and the assimilation of ammonia nitrogen. The difference between the weight of the total residue and the weight of cellulose left was found to represent the amount of mycelium synthesized by the organisms. The mycelium contained 44.3 per cent carbon as determined by analysis of dried fungus mycelium obtained on a sugar medium. Total carbon determinations of the solution gave small and yet definite amounts of carbon other than carbonates in solution. The difference between the amount of ammonia and total nitrogen in solution seems to indicate that, with each period, a greater amount of total nitrogen is found in solution than can be accounted for by the amount of ammonia left in the medium. The difference between these two values represents the organic nitrogen that has

TABLE I.

*Decomposition of Cellulose by Trichoderma sp. in Solution Culture.*

| Incubation. | Carbon transformation. | | | | | | | | | | | | Nitrogen transformation. | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $CO_2$ produced as C. | | | Weight of dry residue. | Cellulose. | | | | Mycelium synthesized. | | C in solution. | | Ammonia N. | | Total N. |
| | | | | | Left. | | Decomposed. | | | | | | | | |
| | Total. | From carbonates. | From cellulose. | | Total. | As C. | Total. | As C. | Total. | As C. | Other than carbonates. | Over control. | Left in solution. | Assimilated. | In solution. |
| *days* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* |
| Control. | 7.4 | 16.2 | | 958.0 | 835.9 | 371.1 | | | | | 3.7 | | 39.2 | | 39.4 |
| 9 | 22.3 | 16.8 | 22.3 | 866.6 | | | | | | | 7.8 | 4.1 | 33.6 | 5.6 | 32.0 |
| 17 | 79.1 | 15.9 | 71.4 | 679.2 | 555.1 | 246.2 | 280.8 | 124.8 | 124.1 | 54.9 | 15.3 | 11.6 | 26.9 | 12.3 | 28.8 |
| 24 | 136.4 | 12.8 | 125.6 | 467.0 | 289.7 | 128.6 | 546.2 | 242.5 | 177.3 | 78.5 | 14.1 | 10.4 | 21.0 | 18.2 | 23.7 |
| 31 | 177.5 | 11.9 | 165.8 | 429.0 | 117.0 | 51.9 | 718.9 | 319.2 | 312.0 | 138.2 | 15.4 | 11.7 | 17.0 | 22.2 | 19.8 |
| 38 | 194.8 | 11.8 | 182.8 | 412.0 | 99.8 | 44.3 | 736.1 | 326.8 | 312.2 | 138.3 | 12.9 | 9.2 | 15.5 | 23.7 | 18.9 |

gone into solution and it increases from 1.9 to 3.4 mg. per culture during the course of incubation. This seems to indicate that a small amount of organic matter passes into solution, in the course of the decomposition of cellulose by *Trichoderma sp.*, probably derived from the protoplasm of the fungus mycelium, either by processes of autolysis or as a result of extraction of the residue during the washing with cold water. The amount of organic matter that goes into solution is rather small, the maximum amounting to less than 4 per cent of the total amount of carbon transformed.

In Table II an attempt is made to account for the products of metabolism in the decomposition of cellulose by *Trichoderma*, based on the results recorded in Table I. By adding the amount of carbon liberated as $CO_2$, the part left in solution, the carbon assimilated by the fungus and built up into its protoplasm, and the carbon left undecomposed in the form of cellulose, practically the total carbon introduced into the cultures in the form of cellulose can be accounted for. There are obviously variations from a complete balance, the extreme variations being +13.0 mg. in the 17 day incubation period, and −17.2 mg. in the next period. However, even these extremes do not constitute more than a 3 to 4 per cent deviation from the theoretical value, which is within the experimental error allowed for these studies. On averaging the figures of the total carbon accounted for experimentally, for the whole period, a value is obtained which is very close to the theoretical.

In the second part of Table II an attempt is made to check up the results obtained for the synthesized fungus mycelium. This was determined, as stated above, experimentally by subtracting the cellulose left in the residue from the total amount of residue. This experiment seems to indicate that $CO_2$ is the only end-product formed in the decomposition of cellulose by *Trichoderma*, in addition to the carbon assimilated by the fungus itself; it is, therefore, possible to determine the amount of carbon assimilated by the fungus, having determined the amount of carbon given off as $CO_2$. Thus the sum of carbon given off as $CO_2$ and the carbon left in solution, subtracted from the amount of carbon of cellulose decomposed, should be equal to the amount of carbon assimilated in the fungus mycelium. In comparing the results obtained on

TABLE II.

*Relation between Cellulose Decomposed in Solution Culture by a Typical Soil Fungus and the Products of Metabolism.**

| Incubation. | Products accounted for. | | | | | Balance between the carbon of cellulose decomposed and the carbon of the products of metabolism. | | | | Relations of metabolic products with cellulose as a source of energy. | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Carbon obtained as. | | | | Total C accounted for. | Cellulose decomposed. | $CO_2$ | In solution. | Carbon unaccounted for directly (assimilated by organism). | Respiration coefficient. | Economic coefficient. | Respiration equivalent. | Plastic equivalent. | Cellulose decomposed to nitrogen assimilated by organism. |
| | $CO_2$ | In solution. | In mycelium. | As undecomposed cellulose. | | | | | | | | | | |
| *days* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg. C* | *mg. C* | *mg. C* | *mg.* | | | *per cent* | *per cent* | |
| 17 | 71.4 | 11.6 | 54.9 | 246.2 | 384.1 | 128.4 | 71.4 | 11.6 | 41.8 | 2.1 | 2.2 | 57 | 44 | 23:1 |
| 24 | 136.4 | 10.4 | 78.5 | 128.6 | 353.9 | 242.5 | 136.4 | 10.4 | 95.7 | 2.6 | 3.1 | 56 | 32 | 30:1 |
| 31 | 165.8 | 11.7 | 138.2 | 51.9 | 367.7 | 319.2 | 165.8 | 11.7 | 141.7 | 1.9 | 2.3 | 52 | 43 | 32:1 |
| 38 | 182.8 | 9.2 | 138.3 | 44.3 | 374.6 | 326.8 | 182.8 | 9.2 | 134.8 | 2.1 | 2.3 | 56 | 42 | 31:1 |
| | | | | | Average. | | | | | | | | | |
| Control. | | | | 371.4 | 370.1 | | | | | | | | | |

* Based upon data recorded in Table I.

this basis with the results obtained previously, a striking similarity is observed. There are, of course, certain variations, for example 54.9 mg. of carbon actually found in the mycelium as against 41.8 mg. obtained by difference in the 17 day incubation period, and 78.5 mg. against 95.7 mg. in the 24 day incubation period. However, the results for the next two periods agree more closely and the variations are undoubtedly due to experimental error in the analyses.

In the last part of Table II the relations of the products of metabolism to each other are reported in numerical ratios (Peterson, Fred, and Schmidt (6)).

$$\text{Respiration coefficient} = \frac{\text{weight of } CO_2 \text{ liberated}}{\text{weight of fungus mycelium}}$$

$$\text{Economic coefficient} = \frac{\text{weight of cellulose decomposed}}{\text{weight of fungus mycelium}}$$

$$\text{Respiration equivalent} = \frac{\text{C of } CO_2 \text{ liberated}}{\text{C of cellulose decomposed}}$$

$$\text{Plastic equivalent} = \frac{\text{C of fungus mycelium}}{\text{C of cellulose decomposed}}$$

The difference between the cellulose in the residue and total residue was taken as the amount of fungus mycelium which was taken to contain 44.3 per cent C. The carbon content of cellulose was 44.4 per cent.

The respiration coefficient or the ratio between the $CO_2$ produced per unit of fungus mycelium synthesized varies from 1.9 to 2.6, giving an average of 2.175, in other words, about twice as much $CO_2$ was given off as there was mycelium synthesized by a fungus utilizing cellulose as the sole source of energy. The economic coefficient gave similar results, with an average of 2.3 parts of cellulose decomposed for every unit of fungus mycelium synthesized. The respiration equivalent, which is the ratio of $CO_2$ produced to cellulose decomposed calculated on the basis of carbon, indicates that on an average 55 per cent of the carbon of the cellulose decomposed is given off as $CO_2$. The plastic equivalent or the ratio between the amount of fungus mycelium synthesized to the amount of cellulose decomposed, calculated on the basis of their carbon content, gave on an average 40 per cent; this indicates that 40 per cent of the carbon of the cellulose decomposed is transformed into fungus mycelium. On adding these last two

values, 95 per cent of the carbon of the cellulose decomposed can be accounted for in the $CO_2$ produced and in the fungus protoplasm synthesized. These results, with cellulose as the sole source of energy, are in conformity with the statement made by Kruse (4) and the results of one of us (8) who used sugars and proteins as energy sources, that fungi assimilate about 40 per cent of the carbon transformed.

The ratio between the amount of cellulose decomposed to that of nitrogen assimilated or transformed from an inorganic form in solution to an organic form in the fungus mycelium are reported in the last column of Table II. These results check up closely with the values obtained previously and reported elsewhere (9). The ratio of 23:1 for the 17 day incubation period is narrower than the average, but it widens with the second and third periods of incubation.

*Experiment 2. Decomposition of Cellulose by Trichoderma sp. in Sand Cultures.*—100 gm. portions of dry, washed, white sand were placed in 300 cc. long necked flasks used in the previous experiment. 1 gm. portions of ground filter paper, 200 mg. of $CaCO_3$, and 20 cc. of a nutrient solution were added to each flask. The solution was of the following composition:

| | |
|---|---|
| $NH_4NO_3$ | 5.0 gm. |
| $K_2HPO_4$ | 3.0 " |
| $MgSO_4 \cdot 7H_2O$ | 2.0 " |
| NaCl | 1.0 " |
| $FeSO_4$ | 0.02 " |
| Distilled water | 1000 cc. |

The flasks were plugged, sterilized at 15 pounds pressure, inoculated with *Trichoderma*, and connected with respiration apparatus in the incubator (25–28°C.). At the end of 1 and 2 weeks incubation, three flasks were disconnected and the contents analyzed. The results reported in Table III represent the averages of the determinations of the contents of the three parallel flasks.

The $CO_2$ produced from the carbonates was deducted from the total amount of $CO_2$ evolved and the difference recorded as "$CO_2$ produced from cellulose." "Cellulose decomposed" represents the difference between the cellulose left in the inoculated flasks and that of the uninoculated controls. "Mycelium synthesized" was calculated from the difference obtained by subtracting the

TABLE III.

*Decomposition of Cellulose by Trichoderma sp. in Sand Culture and the Metabolic Products Formed**

| Incubation. | $CO_2$ produced from cellulose. | | Cellulose decomposed. | | Carbon assimilated. | | Nitrogen assimilated. | | | Cellulose decomposed to nitrogen assimilated. | C/N ratio in synthesized mycellium. | Economic coefficient. | Respiration equivalent. | Plastic equivalent. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Total. | As C. | Total. | As C. | Carbon of cellulose decomposed minus carbon of $CO_2$ liberated. | Mycelium synthesized. | $NH_3$-N | $NO_3$-N | Total. | | | | | |
| *wks.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | | | | *per cent* | *per cent* |
| 1 | 287.0 | 78.0 | 466.0 | 206.9 | 128.9 | 291 | 16.1 | 2.8 | 18.9 | 24:1 | 6.8:1 | 1.6 | 37 | 62 |
| 2 | 461.9 | 125.6 | 921.0 | 408.9 | 283.3 | 640 | 16.0 | 11.4 | 27.4 | 33:1 | 10.3:1 | 1.4 | 30 | 70 |

* All results are averages of three parallel sets of determinations.

carbon liberated as $CO_2$ from the carbon of the cellulose that has been decomposed. Since practically all the carbon of the decomposed cellulose is changed into $CO_2$ or is reassimilated by the organism, such calculation was justified. Knowing the carbon content of the fungus mycelium, the latter can thus be calculated from the amount of carbon assimilated.

The results given in Table III show that the organism utilizes the ammonium radicle in preference to the nitrate. At first only a small amount of the nitrate ion is assimilated, in comparison with the ammonium ion, while during the 2nd week there was no further ammonia assimilation, since all of it had been used up during the 1st week, and the organism had to draw upon the nitrate ion for its nitrogen. There is no reference in the literature concerning the relative value of ammonium and nitrate nitrogen to fungi with cellulose as a source of carbon. There is some evidence, however, that certain fungi will thrive better with $NH_3$·N than with $NO_3$·N. Laurent (5) stated that *Aspergillus niger* grew better with $NH_3$·N, although it could utilize nitrates. Recently Böttger (1) pointed out that nitrates may even be injurious to different microorganisms. It was found that nitrates, in considerable amounts, were injurious to the organisms decomposing cellulose; the injury was specific and not due to the cations or osmotic effects produced by high concentration. It should be pointed out, however, that Hutchinson and Clayton (3) found that the nitrogen requirements of a cellulose-decomposing bacterium could be met both by ammonium salts and nitrates.

The ratio of cellulose decomposed to nitrogen assimilated shows a remarkable similarity to the results obtained in the previous experiment. At the end of the 1st week of incubation, the ratio was narrow (24:1), while at the end of the 2nd week it became 33:1, similar to that found in other studies. The C/N ratio in the synthesized fungus mycelium can readily account for this phenomenon. During the first period of growth, the percentage of nitrogen in the fungus mycelium is higher than during the second period; the economic coefficient or the ratio of cellulose decomposed to the mycelium synthesized was 1.6 for the 1st week and 1.4 for the 2nd. The lower values obtained in the sand experiment from those in the solution experiment indicate that, for the synthesis of fungus protoplasm in the well aerated sand culture, less

cellulose was decomposed than in the poorly aerated solution culture, or that the energy liberated from the decomposition of cellulose in sand media was utilized more economically than in solution media. It can be concluded, therefore, that sand is a better medium, for the growth of the organism, as far as its efficiency of cellulose decomposition is concerned. The respiration equivalent, or the ratio of $CO_2$ produced to cellulose decomposed calculated on the basis of their carbon content, shows a similar relationship. There was considerably less $CO_2$ freed per unit of cellulose decomposed in the sand than in the solution culture, consequently more of the carbon of the cellulose is assimilated by the organism. This ratio was also found higher at the end of the 1st week than of the 2nd, indicating that, in the early part of development, more of the carbon is given off as $CO_2$ than in the later periods. The only contrary assumption would be that more of the carbon of the cellulose is left in the form of intermediary products, such as organic acids; however, the increase in nitrogen assimilation in sand cultures and the fact that these intermediary products could not be demonstrated in more than mere traces speak against such an assumption. The amount of mycelium synthesized per unit of cellulose decomposed bears out these considerations. It is considerably higher than in the solution culture and increases with the period of incubation. The sum of the respiration equivalent and the plastic equivalent is about 100, indicating that all the carbon transformed is accounted for by the $CO_2$ produced and fungus mycelium synthesized.

*Experiment 3. On the Decomposition of Cellulose by Trichoderma sp. in Sterile Soil.*—The previous experiment was repeated using sterilized soil as the medium. 1 gm. portions of ground filter paper and 20 cc. portions of a solution containing 5 gm. of $NH_4NO_3$ and 3 gm. of $K_2HPO_4$ per liter of water were added to 100 gm. portions of soil placed in the long necked flasks, which were then plugged, sterilized at 15 pounds pressure for 2 hours, inoculated, and incubated as before.

The preference of *Trichoderma* for ammonia nitrogen, when cellulose is used as a source of energy, is even more strikingly indicated in the growth of the organism upon sterile soil (Table IV). The maximum amount of nitrate nitrogen assimilated was only a third of the amount assimilated as ammonia. The ratio of cel-

lulose decomposed to nitrogen assimilated is wider in this case than in the previous experiment where sand had been used as a medium. The ratio is 35:1 at the end of the 1st week, rising to 43:1 at the end of the 2nd week, then falling somewhat to 41:1 at the end of the 3rd. This is due to the fact that *Trichoderma* is capable of decomposing the nitrogenous compounds of sterile soil liberating the nitrogen as ammonia, which is then reassimilated in the presence of cellulose as a source of energy. The C/N ratio of the synthesized mycelium follows the same relative course: it is 9.5:1 at the end of the 1st week, 10.2:1 at the end of the 2nd, and 8.8:1 at the end of the 3rd. The narrower C/N ratio found at the end of the 3rd week may be due to the fact that after nearly

TABLE IV.

*Decomposition of Cellulose by Trichoderma sp. in Sterilized Soil.*

| Incubation. | $CO_2$ produced. | | | | Cellulose decomposed. | | C assimilated. | | N assimilated. | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Inoculated sterile soil. | Soil plus cellulose. | From cellulose alone. | Carbon equivalent of $CO_2$ from cellulose. | Total. | As C. | Carbon of cellulose decomposed minus carbon of $CO_2$ liberated. | Mycelium synthesized. | $NH_3$-N | $NO_3$-N | Total. |
| *wks.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | | | | | |
| 1 | 36.7 | 456.7 | 420.0 | 114.2 | 635.0 | 281.9 | 167.7 | 378.0 | 14.7 | 3.2 | 17.9 |
| 2 | 60.7 | 722.2 | 661.5 | 179.9 | 877.0 | 389.4 | 209.5 | 472.0 | 15.3 | 5.1 | 20.4 |
| 3 | 75.3 | 770.2 | 770.2 | 209.5 | 901.0 | 460.0 | 190.5 | 429.0 | 16.1 | 5.6 | 21.7 |

all the cellulose has become decomposed (as seen by the results obtained at the end of the 2nd week), the synthesized protoplasm begins to undergo autolysis giving a higher percentage of nitrogen in the mycelium.

The ratio of the cellulose decomposed to mycelium synthesized (the economic coefficient) rises from 1.7 the 1st to 2.1 the 3rd week. In a similar way the respiration equivalent increases from 40 to 52 indicating that at a later stage of development more of the carbon is given off as $CO_2$ and less of it is assimilated than in the early period of development. No attempt will be made to explain this apparent discrepancy between the soil and sand cultures, due to the reactions involved in the growth of an organism upon the complex soil organic matter.

*Experiment 4. Decomposition of Cellulose by Penicillium sp. in Sand Culture.*—This experiment was an exact duplication of Experiment 2, but using a typical cellulose decomposing soil *Penicillium* in place of *Trichoderma*.

The *Penicillium* was also found to show a preference for the ammonium ion over the nitrate ion (Table VI). The ratio of cellulose decomposed to nitrogen assimilated is essentially the same as that obtained for the *Trichoderma* in the sand culture. It increases from 20:1, on the 5th day, to 35:1, on the 18th day. The ratio of C/N in the mycelium increases from 6.8:1 on the 5th day to 8:7 on the 13th day and drops to 8.0:1 on the 18th day. This is a somewhat narrower ratio, indicating that this organism contains a somewhat higher percentage of nitrogen in its mycelium

TABLE V.

*Relation between Cellulose Decomposed by Trichoderma sp. in Soil Culture and the Products of Metabolism.**

| Incubation. | Ratio of cellulose decomposed to nitrogen assimilated. | C/N ratio in synthesized mycelium. | Economic coefficient. | Respiration equivalent. | Plastic equivalent. |
|---|---|---|---|---|---|
| *wks.* | | | | *per cent* | *per cent* |
| 1 | 35:1 | 9.5:1 | 1.7 | 40 | 59 |
| 2 | 43:1 | 10.2:1 | 1.8 | 46 | 54 |
| 3 | 41:1 | 8.8:1 | 2.1 | 52 | 47 |

* Based upon data recorded in Table IV.

than the *Trichoderma*. The respiration equivalent increases while the plastic equivalent decreases with the period of incubation, showing that at a later stage of development proportionally more of the cellulose decomposed is converted into $CO_2$ than is assimilated in the mycelium.

*Experiment 5. Decomposition of Cellulose by Penicillium sp. in Sterilized Soil.*—This experiment was a duplication of Experiment 3, but with *Penicillium sp.* as the cellulose decomposing agent. In addition to the analyses made in the previous experiments, total carbon determinations were also made on the soil itself and on the soil with the cellulose. The difference between the two gives the total carbon due to the addition of cellulose; this carbon is partly in the form of cellulose and partly as fungus mycelium. By subtracting the amount of carbon found as cel-

TABLE VI.

*Decomposition of Cellulose by Penicillium sp. in Sand Culture and the Products of Metabolism.*

| Incubation. | $CO_2$ produced from cellulose. | | Cellulose decomposed. | | C assimilated. | | N assimilated. | | | Ratio of cellulose decomposed to nitrogen assimilated. | C/N ratio in the mycelium. | Economic coefficient. | Respiration equivalent. | Plastic equivalent. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Total. | As C. | Total. | As C. | Carbon of cellulose decomposed minus carbon of $CO_2$ liberated. | Mycelium synthesized. | $NH_3$-N | $NO_3$-N | Total. | | | | | |
| *days* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | | | | *per cent* | *per cent* |
| 5 | 136.5 | 37.9 | 351.0 | 156.0 | 118.1 | 266.0 | 14.3 | 2.9 | 17.2 | 20:1 | 6.8:1 | 1.3 | 24 | 75 |
| 9 | 341.4 | 93.3 | 580.0 | 257.6 | 164.3 | 371.0 | 16.1 | 3.4 | 19.5 | 30:1 | 8.4:1 | 1.5 | 37 | 63 |
| 13 | 406.2 | 111.0 | 648.0 | 287.6 | 176.6 | 396.0 | 15.1 | 5.1 | 20.2 | 32:1 | 8.7:1 | 1.6 | 38 | 61 |
| 18 | 519.0 | 141.8 | 649.0 | 288.1 | 146.3 | 330.0 | 14.7 | 3.7 | 18.4 | 35:1 | 8.0:1 | 1.9 | 49 | 50 |

lulose from the total carbon found in the soil the amount of carbon assimilated by the organism is obtained.

[Carbon in soil + carbon in cellulose] − [carbon of original soil + carbon left as cellulose] = carbon of synthesized mycelium.

The results thus obtained can serve as a check upon the amount of carbon obtained by subtracting the carbon of the $CO_2$ from the carbon of the cellulose decomposed, as done in the previous experiments.

Cellulose in control − cellulose left in soil = cellulose decomposed.
Carbon of cellulose decomposed − carbon of $CO_2$ liberated = carbon of synthesized mycelium.

The actual results show that both methods of calculating the carbon assimilated by the organism check very closely (Table VII). The quantity of carbon obtained by the first and second methods of calculation are, for the 1st week, 103.0 and 118.0 mg., for the 2nd week, 223.1 and 216.0 mg., and, for the 3rd week, 185.0 and 193.0 mg. of carbon respectively. The maximum divergence between the two methods was 15 mg. during the 1st week and only 7 and 8 mg. during the 2nd and 3rd weeks, respectively. The first method; *e.g.*, the one derived by subtracting the carbon left in the form of cellulose from the total carbon of the soil, was taken as the basis for calculation of fungus synthesized, allowing 44.3 per cent as the carbon content of the fungus mycelium. The amount of mycelium was found in this experiment to decrease also after the 2nd week, probably due to autolytic phenomena, in a manner similar to those observed in the growth of *Trichoderma* in the soil.

The studies on the nitrogen transformation bring out the same general relations. As in all the previous cases the nitrate ion was utilized only to a small extent, the organism preferring the nitrogen of the ammonium ion. The ratio of cellulose decomposed to nitrogen assimilated is also about the same as in the previous cases, 26:1 at the end of the 1st week and 35:1 at the end of the 2nd and 3rd weeks (Table VIII).

In accounting for the products of the decomposition of cellulose the sum of the carbon in $CO_2$ produced, cellulose left, and mycelium synthesized is found to be nearly equal to the carbon found as

TABLE VII.

*Decomposition of Cellulose by Penicillium sp. Grown in Sterilized Soil.*

| Incubation. | Carbon liberated as $CO_2$ from 100 gm. of soil. | | | Cellulose in 100 gm. of soil. | | | | Total carbon in 100 gm. of soil. | | | Balance of carbon assimilated. | | | Nitrogen transformation. | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Left. | | Decomposed. | | | | | | | | $NH_3$-N | | | $NO_3$-N | | | | |
| | Soil itself. | Soil plus cellulose. | Cellulose. | Total. | As C. | Total. | As C. | In 100 gm. of soil. | In 100 gm. of soil plus cellulose. | As cellulose plus fungus mycelium. | Total carbon minus carbon left as cellulose. | Cellulose decomposed minus carbon liberated as $CO_2$ | Mycelium synthesized. | In soil itself. | In soil plus cellulose. | Assimilated by organism. | In soil itself. | In soil plus cellulose. | Assimilated by organism. | Total nitrogen assimilated. |
| *wks.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* |
| 1 | 25.9 | 77.0 | 51.1 | 539.0 | 239.0 | 381.0 | 169.1 | 1348 | 1690 | 342 | 103.3 | 118.0 | 232 | 21.2 | 9.0 | 12.2 | 18.3 | 16.0 | 2.3 | 14.5 |
| 2 | 41.6 | 181.7 | 140.1 | 117.0 | 51.9 | 803.0 | 356.5 | 1295 | 1570 | 275 | 223.1 | 216.0 | 503 | 23.8 | 5.2 | 18.6 | 17.6 | 13.4 | 4.2 | 22.8 |
| 3 | 45.4 | 207.6 | 162.2 | 120.0 | 53.0 | 800.0 | 355.2 | 1327 | 1565 | 238 | 185.0 | 193.0 | 417 | 22.7 | 4.4 | 18.3 | 18.4 | 14.3 | 4.1 | 22.4 |
| Control. | | | | 920.0 | 408.5 | | | | | | | | | | | | | | | |

cellulose in the control uninoculated flask. Thus a total of 393.1, 415.1, and 400.7 mg. of carbon is obtained for the 1st, 2nd, and 3rd weeks, respectively, against 408.5 mg. which is the amount of carbon found in the cellulose of the uninoculated control. The variations from the expected sum are for the 1st week's incubation period −15.4 mg., for the 2nd week +6.6 mg., and for the 3rd week −7.8 or an extreme variation of about 3 per cent. The average for the three periods comes even closer to the expected value.

The respiration equivalent increases while the plastic equivalent decreases with the period of incubation and the sum of the two varies from 90 to 98. This shows more clearly that the products

TABLE VIII.

*Relation between Cellulose Decomposed by Penicillium sp. in Soil Culture and the Products of Metabolism.**

| Incubation. | Sum of the products accounted for. | | | | Relations of metabolism products. | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | C contained in. | | | | | | | | |
| | $CO_2$ | Cellulose left. | Mycelium. | Total C accounted for. | Cellulose decomposed to N assimilated. | C/N ratio in mycelium. | Economic coefficient. | Respiration equivalent. | Plastic equivalent. |
| *wks.* | *mg.* | *mg.* | *mg.* | *mg.* | | | | *per cent* | *per cent* |
| 1 | 51.1 | 239.0 | 103.0 | 393.1 | 26:1 | 7:1 | 1.6 | 30 | 60 |
| 2 | 140.1 | 51.9 | 223.1 | 415.1 | 35:1 | 10:1 | 1.6 | 39 | 52 |
| 3 | 162.2 | 53.0 | 185.0 | 400.7 | 35:1 | 8:1 | 1.9 | 46 | 52 |
| Control. | | | | 403.0 | | | | | |

*Based upon data recorded in Table VII.

of the decomposition of cellulose are only $CO_2$ and synthesized fungus mycelium; the sum of these two accounts, within the experimental error, for the total amount of cellulose decomposed.

## GENERAL DISCUSSION.

The experiments described above are of interest from the point of view of both the biochemistry of fungi and soil fertility; they point definitely to the conclusion that, in the decomposition of cellulose by the two fungi studied, $CO_2$ and fungus mycelium are the only end-products formed, while no intermediate and no other final products are left in the medium. These fungi utilize the cellulose

as a source of energy for the synthesis of their own protoplasm. Thus a part of the carbon contained in the cellulose is reassimilated, while the remainder is given off as $CO_2$. The relationship between these two products varies with the age of the organisms with the evidence pointing toward a greater proportion of assimilation in the earlier stages. The nitrogen transformation by the organism is a result of the carbon transformation: there is a definite correlation between the amount of carbon assimilated and the amount of nitrogen required for the synthesis of the fungus protoplasm, varying with age and type of organism. At the earlier stages of growth the protoplasm contains a higher percentage of nitrogen and thus decomposes less cellulose per unit of nitrogen assimilated. During the stage of active growth, the percentage of nitrogen in the protoplasm falls, thus enabling the organism to decompose greater amounts of cellulose per unit of nitrogen assimilated. When the period of active growth is completed, autolysis begins to take place, with a consequent rise of the percentage of nitrogen in the mycelium, due largely to the decomposition of the carbonaceous reserve products of the protoplasm.

To arrive at the amount of fungus mycelium synthesized in soil and sand, an indirect method had to be used. Even in solution culture it is not possible to separate the growth from the cellulose and determine it separately. Thus that portion of carbon which has neither been given off as $CO_2$ nor left as cellulose, has been taken to represent the amount that has been reassimilated. The accuracy of this method has been confirmed by direct analyses of the total carbon in the various fractions.

In the decomposition of cellulose by fungi the only residual substance is thus found to be the fungus protoplasm. This phenomenon has an important bearing not only on the decomposition of cellulose in the soil but also upon the transformation of nitrogen in the soil, resulting in the storing away of considerable quantities of available nitrogen into an unavailable form, in direct proportion to the amount of cellulose available. It has also a direct bearing upon the formation of humus in the soil as will be shown later. Of course the synthesized fungus mycelium will be further decomposed in normal soils by other microorganisms, resulting in the liberation of nitrogen in an available form.

SUMMARY.

The carbon and nitrogen transformations in the decomposition of cellulose by two typical soil fungi, a *Trichoderma* and a *Penicillium*, have been studied. It has been found that cellulose is completely decomposed by these organisms, giving $CO_2$ as the only waste product. No intermediary products are left in the medium. A considerable part of the carbon of the cellulose is reassimilated by these organisms and built into protoplasm. These results have been obtained both in a direct and an indirect way. It has been conclusively shown that the carbon evolved as $CO_2$ and the carbon assimilated by the fungi account for nearly all of the carbon of the cellulose decomposed. The divergencies are small and are due to experimental error. The carbon and nitrogen asssimilated by the organisms show a definite relationship to one another. The organisms prefer ammonia to nitrate as a source of nitrogen. This is assimilated and transformed into microbial protoplasm. A direct correlation is thus found between the amount of cellulose decomposed and the amount of nitrogen transformed into an insoluble organic form.

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# THE STATE OF THE SERUM CALCIUM IN EXPERIMENTAL HYPO- AND HYPERCALCEMIA.

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The calcium of the blood serum may be separated into two portions by passing the serum through a semipermeable membrane, impervious to colloids. This observation was first made by Rona and Takahashi (1) and has been repeatedly confirmed. That portion of the calcium retained by the membrane is spoken of as the colloidal, and that which passes through the membrane, the diffusible serum calcium.

In this regard the calcium differs from the chloride, phosphate, sodium, and potassium of the blood serum which, according to Neuhausen and Pincus (2), are totally diffusible. Cushny (3) suggested that the combined state of the serum calcium might account for its excretion by the intestinal epithelium and various observations have been made on the state of the serum calcium in the event of disordered calcium metabolism. Von Meysenbug and McCann (4), in a study of four cases of experimental parathyroid tetany in dogs and in two cases of human rickets, concluded that the ratio between the colloidal and diffusible serum calcium remains constant when the total amount is reduced. Cruickshank (5) observed that in severe parathyroid tetany in dogs, the decrease is chiefly in the colloidal calcium and that while in the serum of normal animals the diffusible calcium averages from 60 to 70 per cent, it reaches as high as 94 per cent in tetany. Salvesen and Linder (6), in a study of the relation between calcium and protein of serum in parathyroid tetany concluded that the tetany is probably due to a primary decrease in the diffusible (and ionized) fraction.

The purpose of this investigation is to make repeated observations on the diffusible-colloidal serum calcium ratio during the development and course of experimental hypo- and hypercalcemia.

## EXPERIMENTAL.

The diffusible and colloidal serum calcium were separated by the method described by Moritz (7), modified slightly in that the

TABLE I.

*Changes in the State of the Serum Calcium of Rabbits Incident to Frequent Bleeding.*

| Animal No. | Time of bleeding. | | Serum calcium. | | Change in amount of calcium. | |
|---|---|---|---|---|---|---|
| | Date. | Hr. | Diffusible. | Colloidal. | Diffusible. | Colloidal. |
| | | | *mg. per cent* | *mg. per cent* | *per cent* | *per cent* |
| 275 | June 23 | 9.00 a.m. | 7.2 | 5.4 | 0 | 0 |
| | | 4.00 p.m. | 6.5 | 5.2 | −10 | −4 |
| | | 11.00 " | 7.2 | 4.7 | 0 | −13 |
| | " 24 | 6.00 a.m. | 6.6 | 5.5 | −8 | 2 |
| | | 1.00 p.m. | 7.2 | 5.4 | 0 | 0 |
| | | 8.00 " | 6.5 | 5.4 | −10 | 0 |
| | " 25 | 2.00 a.m. | 6.8 | 5.6 | −6 | 2 |
| | | 9.00 " | 7.6 | 5.3 | 5 | −2 |
| 278 | " 13 | 10.00 " | 6.8 | 5.3 | 0 | 0 |
| | | 5.00 p.m. | 6.5 | 4.8 | −4 | −9 |
| | | 12.00 " | 5.9 | 5.2 | −13 | −2 |
| | " 14 | 7.00 a.m. | 6.8 | 5.6 | 0 | 6 |
| | | 2.00 p.m. | 5.7 | 5.1 | −16 | −4 |
| | | 9.00 " | 6.6 | 4.9 | −3 | −7 |
| | " 15 | 4.00 a.m. | 7.1 | 5.0 | 4 | −6 |
| | | 11.00 " | 6.3 | 4.6 | −7 | −13 |

TABLE II.

*Changes in the State of the Serum Calcium of a Rabbit Following Ether Anesthesia.*

| Animal No. | Time of bleeding. | | Serum calcium. | | Change in amount of calcium. | |
|---|---|---|---|---|---|---|
| | Date. | Hr. | Diffusible. | Colloidal. | Diffusible. | Colloidal. |
| | | | *mg. per cent* | *mg. per cent* | *per cent* | *per cent* |
| 277 | June 23 | 8.00 a.m. | 6.3 | 4.7 | 0 | 0 |
| | | 8.30 " | Ether anesthesia for 30 min. | | | |
| | | 3.00 p.m. | 5.6 | 4.2 | −11 | −11 |
| | | 10.00 " | 6.2 | 4.2 | −2 | −11 |
| | " 24 | 5.00 a.m. | 5.5 | 4.3 | −13 | −8 |
| | | 12.00 " | 6.4 | 4.7 | 2 | 0 |
| | | 7.00 p.m. | 5.7 | 4.8 | −10 | 2 |

filtrate was collected in 3 cc. rather than 5 cc. of distilled water. Since the colloidal as well as the diffusible calcium was determined, the residuum was pipetted out of the collodion sac at the end of the period of filtration, the sac washed three times with 1 cc. of distilled water, using the same pipette throughout. The residuum together with the washings constituted the colloidal serum calcium.

Calcium determinations were made by the Tisdall (8) modification of the Kramer-Tisdall method. It was found advisable to allow the serum to stand 1 hour after the addition of ammonium oxalate to insure complete precipitation of the calcium oxalate. As nearly as possible, absolute uniformity of method was observed throughout.

Sixteen rabbits weighing between 2.0 and 2.4 kilos were used. Hypocalcemia was produced in seven of them by thyroparathyroidectomy and hypercalcemia in six by the subcutaneous injection of parathyroid extract (Collip and coworkers (9)), supplied through the courtesy of Eli Lilly and Company. Three rabbits served as controls. 5 cc. of blood were withdrawn from the ear vein at each bleeding and calcium determinations made on the diffusible and colloidal portions of the serum. Percentage variations of each portion from the amounts determined in the first bleeding are indicated in the tables and the difference between the diffusible and colloidal serum calcium indicates the degree of alteration of their ratio. All rabbits were supplied with an abundance of lettuce, oats, and water before and during the course of the experiment.

Rabbits 275 and 278 were bled at 7 hour intervals and Table I shows that the maximum difference between diffusible and colloidal calcium was 13 per cent. This figure represents the error of the method as well as the actual change in the diffusible-colloidal ratio during the course of the experiment.

Rabbit 277 was subjected to ether anesthesia for 30 minutes following the first bleeding and serves as a control on the rabbits which were thyroparathyroidectomized. Table II shows that the maximum change in the diffusible-colloidal ratio was 12 per cent.

Thyroparathyroidectomy was performed on seven animals, following the technique described by Ecker and Goldblatt (10),

TABLE III.

*Changes in the State of the Serum Calcium of Rabbits Following Thyroparathyroidectomy.*

| Animal No. | Time of bleeding. | | Serum calcium. | | Change in amount of calcium. | |
|---|---|---|---|---|---|---|
| | Date. | Hr. | Diffusible. | Colloidal. | Diffusible. | Colloidal. |
| | | | *mg. per cent* | *mg. per cent* | *per cent* | *per cent* |
| 270 | June 9 | 8.00 a.m. | 6.5 | 5.7 | 0 | 0 |
| | | 9.00 " | Thyroparathyroidectomy. | | | |
| | | 7.00 p.m. | 5.2 | 5.2 | −20 | −9 |
| | " 10 | 10.00 a.m. | 3.8 | 4.5 | −41 | −21 |
| | | 5.00 p.m. | 4.1 | 3.6 | −37 | −37 |
| | | 12.00 " | 3.4 | 2.8 | −48 | −51 |
| | " 11 | 11.00 a.m. | 3.5 | 3.4 | −46 | −40 |
| 138 | May 12 | 10.00 " | 8.0 | 5.1 | 0 | 0 |
| | | 11.00 " | Thyroparathyroidectomy. | | | |
| | | 6.00 p.m. | 6.0 | 4.5 | −25 | −12 |
| | " 13 | 1.00 a.m. | 4.9 | 4.1 | −39 | −20 |
| | | 7.00 " | 3.7 | 3.9 | −54 | −24 |
| 264 | June 5 | 8.00 " | 7.6 | 6.4 | 0 | 0 |
| | | 8.30 " | Thyroparathyroidectomy. | | | |
| | | 1.30 p.m. | 5.8 | 4.4 | −24 | −31 |
| | " 6 | 7.30 a.m. | 4.4 | 3.5 | −42 | −45 |
| | | 1.30 p.m. | 6.3 | 4.0 | −17 | −37 |
| 274 | " 20 | 8.00 a.m. | 7.3 | 5.4 | 0 | 0 |
| | | 8.30 " | Thyroparathyroidectomy. | | | |
| | | 3.30 p.m. | 4.7 | 4.2 | −36 | −12 |
| | | 10.30 " | 4.5 | 4.3 | −38 | −20 |
| | " 21 | 7.30 a.m. | 4.9 | 5.8 | −33 | 7 |
| 276 | " 20 | 8.30 " | 6.3 | 5.5 | 0 | 0 |
| | | 9.00 " | Thyroparathyroidectomy. | | | |
| | | 4.00 p.m. | 3.3 | 4.2 | −48 | −25 |
| | | 11.00 " | 3.3 | 4.0 | −48 | −27 |
| | " 21 | 8.00 a.m. | 5.2 | 5.4 | −17 | −2 |
| 273 | " 9 | 9.00 " | 7.4 | 5.7 | 0 | 0 |
| | | 9.30 " | Thyroparathyroidectomy. | | | |
| | | 7.30 p.m. | 4.9 | 5.2 | −35 | −9 |
| | " 10 | 10.30 a.m. | 3.9 | 4.2 | −47 | −26 |
| | | 4.30 p.m. | 4.3 | 4.0 | −42 | −30 |
| | | 11.30 " | 3.7 | 3.3 | −50 | −42 |

TABLE III—*Concluded.*

| Animal No. | Time of bleeding. | | Serum calcium. | | Change in amount of calcium. | |
|---|---|---|---|---|---|---|
| | Date. | Hr. | Diffusible. | Colloidal. | Diffusible. | Colloidal. |
| | | | *mg. per cent* | *mg. per cent* | *per cent* | *per cent* |
| | June 11 | 11.30 a.m. | 4.1 | 3.5 | −45 | −38 |
| | | 6.30 p.m. | 4.3 | 3.6 | −42 | −37 |
| 266 | " 5 | 8.30 a.m. | 7.3 | 5.6 | 0 | 0 |
| | | 9.00 " | Thyroparathyroidectomy. | | | |
| | " 6 | 10.30 " | 4.3 | 3.9 | −41 | −30 |
| | | 4.30 p.m. | 4.6 | 3.6 | −37 | −36 |
| | | 11.30 " | 6.2 | 4.3 | −15 | −23 |

and changes in the serum calcium are recorded in Table III. Ether anesthesia was used and the average duration of the operation was about 30 minutes. Rabbit 274 developed tetany

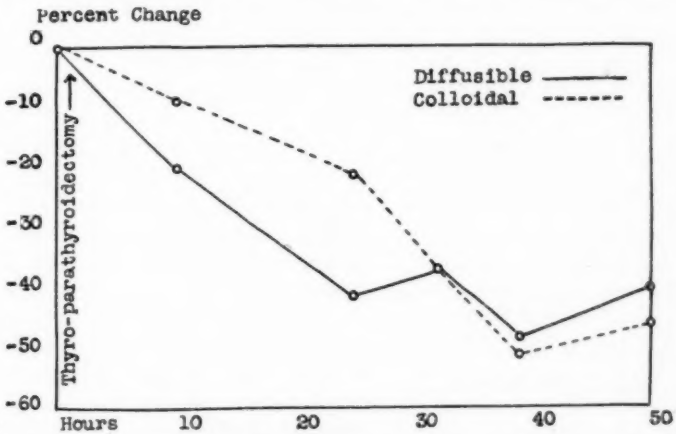


CHART 1. Rabbit 270. Changes in the diffusible and colloidal serum calcium of a rabbit following thyroparathyroidectomy.

in 10 hours after the operation and died 26 hours postoperative. No. 138 developed tetany in 15 hours and died 24 hours postoperative. All other animals except No. 266, which was appar-

TABLE IV.

*Changes in the State of the Serum Calcium of Rabbits Following the Subcutaneous Injection of Parathyroid Extract.*

| Animal No. | Time of bleeding. | | Time of injection. | | Amount (units). | Serum calcium. | | Change in amount of calcium. | |
|---|---|---|---|---|---|---|---|---|---|
| | Date. | Hr. | Date. | Hr. | | Diffusible. | Colloidal. | Diffusible. | Colloidal. |
| | | | | | | *mg. per cent* | *mg. per cent* | *per cent* | *per cent* |
| 265 | June 23 | 11.00 a.m. | June 23 | 11.30 p.m. | 10–P 5 | 7.8 | 6.1 | 0 | 0 |
| | | | " 24 | 1.30 a.m. | 10–P 5 | | | | |
| | | | | 4.30 " | 10–P 20 | | | | |
| | | | | 7.30 " | 10–P 20 | | | | |
| | " 24 | 9.00 " | | 9.30 " | 10–P 20 | 8.4 | 6.5 | 8 | 6 |
| | " 24 | 12.30 p.m. | | | | 9.4 | 7.4 | 20 | 21 |
| | " 25 | 8.30 " | | | | 6.9 | 6.1 | −12 | 0 |
| 260 | May 12 | 10.30 a.m. | May 12 | 10.45 " | 10–P 5 | 6.6 | 6.3 | 0 | 0 |
| | | | | 1.00 p.m. | 10–P 5 | | | | |
| | | | | 4.00 " | 10–P 5 | | | | |
| | | | | 7.00 " | 10–P 5 | | | | |
| | | | | 10.00 " | 10–P 5 | | | | |
| | " 13 | 12.30 " | | | | 8.0 | 7.9 | 21 | 25 |
| | | 4.30 " | | | | 9.7 | 8.5 | 47 | 35 |
| 261 | " 12 | 10.40 " | " 12 | 11.00 a.m. | 10–P 5 | 6.7 | 7.2 | 0 | 0 |
| | | | | 1.00 p.m. | 10–P 5 | | | | |
| | | | | 4.00 " | 10–P 5 | | | | |
| | | | | 7.00 " | 10–P 5 | 7.7 | 7.4 | 15 | 3 |
| | | 8.40 p.m. | | 10.00 " | 10–P 5 | | | | |
| | " 13 | 6.40 a.m. | | | | 9.0 | 7.8 | 34 | 8 |
| | | 4.40 p.m. | | | | 6.8 | 7.0 | 2 | −3 |
| 278 | " 20 | 9.00 a.m. | | | | 6.3 | 4.7 | 0 | 0 |
| | | | " 20 | 11.00 a.m. | 10–P 20 | | | | |
| | | | | 1.00 p.m. | 10–P 20 | | | | |
| | | 3.00 p.m. | | 3.00 " | 10–P 20 | 7.4 | 4.2 | 17 | −10 |
| | | | | 5.00 " | 10–P 20 | | | | |
| | | 10.30 " | | 7.00 " | 10–P 20 | 8.1 | 5.3 | 29 | 13 |
| | " 21 | 8.30 a.m. | | | | 6.8 | 5.1 | 8 | 9 |
| 263 | " 25 | 9.00 " | " 25 | 9.00 a.m. | 10–P 5 | 7.1 | 5.6 | 0 | 0 |
| | | | | 12.00 p.m. | 10–P 5 | | | | |

TABLE IV—*Concluded.*

| Animal No. | Time of bleeding. | | Time of injection. | | Amount (units). | Serum calcium. | | Change in amount of calcium. | |
|---|---|---|---|---|---|---|---|---|---|
| | Date. | Hr. | Date. | Hr. | | Diffusible. | Colloidal. | Diffusible. | Colloidal. |
| | | | | | | *mg. per cent* | *mg. per cent* | *per cent* | *per cent* |
| 263 | May 25 | | May 25 | 3.00 p.m. | 10–P 5 | | | | |
| | | | | 6.00 " | 10–P 5 | 10.2 | 6.8 | 44 | 21 |
| | | 7.00 p.m. | | 9.00 " | 10–P 5 | | | | |
| | | 12.00 " | | 12.00 " | 10–P 5 | 10.4 | 7.5 | 47 | 34 |
| | " 26 | 3.00 " | | | | 6.6 | 6.3 | −7 | 12 |
| 259 | " 27 | 9.00 " | " 27 | 10.00 a.m. | 10–P 20 | 6.6 | 5.1 | 0 | 0 |
| | | | | 1.00 p.m. | 10–P 20 | | | | |
| | | | | 4.00 " | 10–P 20 | | | | |
| | | 7.00 p.m. | | 7.00 " | 10–P 20 | 7.3 | 5.5 | 10 | 8 |
| | | | | 10.00 " | 10–P 20 | | | | |
| | " 28 | 1.00 a.m. | | | | 8.1 | 6.5 | 23 | 27 |
| | | 6.00 " | | | | 7.9 | 5.6 | 20 | 10 |
| | | 6.00 p.m. | | | | 6.8 | 4.9 | 3 | −4 |

ently normal at all times, manifested varying degrees of nervous irritability and tremor, although all of them recovered.

During the rapid fall in the amount of serum calcium it is seen that the diffusible fraction falls more rapidly than does the colloidal in all animals except No. 264. The differences observed are greater than the variation occurring in control animals. It may be seen that except in Rabbits 274 and 138, which died in tetany, and No. 264, the original ratio between diffusible and colloidal calcium showed a tendency to become reestablished.

Chart 1 illustrates graphically the changes in the diffusible-colloidal serum calcium ratio following thyroparathyroidectomy.

Table IV records the changes in the state of the serum calcium following the subcutaneous injection of parathyroid extract. Parathyroid extract was administered in the manner recommended by Collip and coworkers (9) to obtain the greatest rise in serum calcium. In Rabbits 261, 263, and 278 the diffusible calcium rose relatively faster than the colloidal. In Nos. 265, 260,

and 259 no significant alteration of the diffusible-colloidal ratio was observed.

SUMMARY.

Of the three control animals observed, one of which was subjected to ether anesthesia for 30 minutes, the variation in the ratio between diffusible and colloidal serum calcium did not exceed 13 per cent, nor was there any consistent alteration of the ratio. Of seven animals which developed hypocalcemia following thyroparathyroidectomy, six manifested a relatively more rapid fall in the diffusible than the colloidal serum calcium. Four of those six recovered and in them the original diffusible-colloidal serum calcium ratio tended to become reestablished with the beginning of the rise of serum calcium. The diffusible-colloidal calcium ratio of the two that died in tetany was not restored.

Rabbits responded less readily than dogs to the administration of parathyroid extract, requiring much larger injections per kilo of body weight than were used by Collip to obtain the same rise in dogs. In three rabbits the diffusible-colloidal serum calcium ratio was not disturbed by the rise in total serum calcium following the administration of parathyroid extract. In three other rabbits the diffusible fraction rose relatively more rapidly than did the colloidal.

This investigation indicates that in a general way a decrease in serum calcium such as occurs following thyroparathyroidectomy shows a disproportionately great decrease of the diffusible fraction. But in an increase in serum calcium produced by injection of parathyroid extract (Collip) the ratio of the two did not show consistent changes.

I wish to acknowledge my sincere thanks to Professor H. T. Karsner for very helpful suggestions and criticisms during this investigation and to Doctor Harry Goldblatt for his unfailing interest and assistance.

I also wish to thank Eli Lilly and Company for the parathyroid extract they so willingly supplied.

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# BLOOD CHANGES DURING DIGESTION WITH SPECIAL REFERENCE TO UREA FORMATION.

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Bollman, Mann, and Magath (1) have recently published what appears to be incontrovertible experimental evidence that the liver is the chief site of urea formation in the body. They give an extensive review of the literature dealing with the subject of urea formation, and it will not be necessary to restate the problem here.

It is well to recall, however, that Van Slyke (2) in an extensive study of the amino acids came to the conclusion that the liver is not only the organ most active in absorbing amino acids from the blood stream but also in altering them in such a way as to prepare them for the formation of urea or for storage in the body as protein.

We must also refer to the important contribution of Folin and Berglund (3), who have studied the changes in the urea and amino acid nitrogen of the blood in human subjects during the process of food absorption. They advance an argument which runs as follows:

"To prove that the liver is even the predominant place for deamination and urea formation it is necessary to show that during the early stages of absorption of amino-acids the increase in the urea content of the blood *precedes* the increase in its content of amino-acids. If the increase in the amino-acid content precedes that of the urea . . . . this does not conclusively prove that the liver is not the special seat of the formation, because the urea-forming process may be very much slower than the absorption process under such unusual conditions."

It is clear, therefore, that a preliminary rise in amino acid nitrogen would not necessarily preclude the possibility of the liver being the specialized organ for deamination and urea formation, whereas the rise in urea nitrogen preceding a rise in amino N would furnish positive proof of the liver's important rôle as an organ of urea formation. Folin and Berglund in their paper show that this first requirement of a theory of the liver's urea-forming function is not sustained by the results of their experiments.

In a previous investigation (4) we found it necessary to study blood changes during digestion and the results obtained with well nourished and with fasted dogs serve as a demonstration of the proof Folin and Berglund postulate as essential to any hypothesis of the liver's predominant part in urea formation. Our study of the blood changes shows that the urea N of the blood rises either before there is an appreciable increase in the blood amino acids, or, when the rise is concomitant, the urea nitrogen increase is much more striking. Although the experiments of Bollman, Mann, and Magath furnish practically incontrovertible evidence for the preponderant, if not actually exclusive, urea-forming function of the liver, it seemed to us that our confirmation findings are of some value.

## EXPERIMENTAL.

Our earlier experiments have all been performed on dogs which received a mixed diet containing moderate amounts of meat. Since those first experiments could be objected to on the ground that the early rise in the urea N of the blood was due to the absorption from the intestinal tract of preformed urea derived from the meat, we modified the experimental procedure by substituting a synthetic food preparation (Dryco) which is rich in protein[1] and used widely for infant feeding. This commercial article has a definite composition and the food was thus uniform in all our tests. The Dryco powder, suspended in a measured quantity of water, was given to the dogs by stomach tube. It may be mentioned that the results obtained by feeding Dryco

[1] Dryco is an artificial dry milk preparation of the following composition: 32 per cent protein, 12 per cent butter fat, 46 per cent milk sugar, and 7 per cent salts.

were practically identical with those found in the experiments with the meat diet, but we are omitting nearly all of the meat experiments to avoid the criticism that the preliminary rise in the urea nitrogen of the blood resulted from absorption of preformed urea present in the food.

The experiments were always performed 24 hours after the last feeding; *i.e.*, when the process of absorption due to previous food consumption had already been completed. A sample of blood was taken before passing the stomach tube (this sample is marked in the records as the 0 time blood), whereupon the food was introduced into the stomach. The entire operation was completed within 5 minutes. The collection of further blood samples was timed from that moment. The blood was drawn directly from the heart and represents, therefore, at all times the mixed systemic blood. The bloods were precipitated immediately, and all the analyses were made in the course of the day. It was our intention to study the urinary output of nitrogenous products together with the blood changes, but this was not always practicable. With several dogs we succeeded in obtaining also the urines, and the data obtained in those experiments are included in Table I. It should be stated that the urines were collected quantitatively by aspirating through a catheter and washing the urinary bladder thoroughly with distilled water. The urine samples were generally taken at three intervals covering the periods from 0 to 3, 3 to 5, and from 5 to 9 hours after feeding. The analytical procedures employed in this study were all according to the Folin Manual (5).

The results presented in Table I show, in the case of Dog 7, a certain uniformity in the blood changes both in the prefasting and in the postfasting condition. In the first two experiments of this series a moderate amount of meat was ingested, and, by the end of 3 hours after the digestive process commenced, the amino acid nitrogen of the blood had risen to a maximum value of 9.4 mg. per 100 cc. of blood. The urea N curve, however, rises much more steeply at the same time. The steep rise in the urea N is especially noticeable in the third experiment which was performed on the same dog but after it had fasted 16 days. In the last two experiments, the same quantity of Dryco having been administered by stomach tube, the difference in the rate

TABLE I.

| Dog No. | Hrs. after feeding. | Blood analysis. | | | Urine analysis. | | | | Remarks. |
|---|---|---|---|---|---|---|---|---|---|
| | | Non-protein N. | Urea N. | $NH_2$ N | Total N. | Urea N. | $NH_3$ N | $NH_2$ N | |
| | | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg.* | *mg.* | *mg.* | *mg.* | |
| | 0 | 29.4 | 11.4 | 7.3 | | | | | Well nourished. 400 gm. ground chuck meat, 64 gm. sucrose. |
| | 1.5 | 34.6 | 15.6 | 8.7 | | | | | |
| | 3 | 44.7 | 18.8 | 9.5 | 340 | 287 | 7 | 1.9 | |
| | 5 | 42.2 | 22.4 | 9.4 | | | | | |
| | 9 | 40.8 | 17.8 | 9.3 | 8969 | 7129 | 278 | 50 | |
| | 0 | 30.1 | 11.0 | 8.4 | | | | | Well nourished. 230 gm. Dryco by tube (74 gm. protein). |
| | 3 | 34.9 | 12.9 | 9.4 | 1492 | 728 | 24 | 17 | |
| 7 | 5 | 35.3 | 11.8 | 8.4 | 728 | 664 | 3 | 8 | |
| | 9 | 37.0 | 13.0 | 8.8 | 1613 | 1410 | 29 | 20 | |
| | 0 | 38.7 | 15.6 | 10.2 | | | | | Fasted 16 days. Lost 26 per cent. 230 gm. Dryco by tube. |
| | 1.5 | 46.9 | 24.4 | 11.0 | | | | | |
| | 3 | 43.5 | 23.9 | 10.3 | 1205 | 1108 | 16 | 7 | |
| | 5 | 45.0 | 22.2 | 10.7 | 926 | 821 | 12 | 5 | |
| | 9 | 44.4 | 23.9 | 9.9 | 1886 | 1662 | 19 | 13 | |
| | 0 | 54.0 | 25.8 | 8.7 | | | | | Well nourished. 300 gm. ground chuck meat, 48 gm. sucrose. |
| | 1.5 | 57.7 | 26.8 | 7.7 | | | | | |
| | 3 | 54.5 | 27.7 | 8.1 | 1530 | 1265 | 45 | 12.7 | |
| | 5 | 53.1 | 26.0 | 9.4 | 875 | 684 | 23 | 7.5 | |
| | 9 | 58.2 | 24.2 | 9.8 | 1552 | 1286 | 47 | 12.5 | |
| | 0 | 42.5 | 20.5 | 8.5 | | | | | Fasted 14 days. Lost 17 per cent. 300 gm. meat, 48 gm. sucrose. |
| | 1.5 | 51.3 | 23.1 | 7.9 | | | | | |
| 8 | 3 | 58.8 | 25.9 | 8.7 | 702 | 568 | 16.5 | 11.9 | |
| | 5 | 64.5 | 31.6 | 9.1 | 834 | 638 | 18 | 4.6 | |
| | 9 | 63.8 | 34.6 | 11.5 | 1887 | 1633 | 55 | 11.6 | |
| | 0 | 45.1 | 21.4 | 8.4 | | | | | Dog has recovered prefasting weight. 212 gm. Dryco by tube. |
| | 1.5 | 47.6 | 25.4 | 9.0 | | | | | |
| | 3 | 52.4 | 28.6 | 9.3 | | | | | |
| | 5 | 52.2 | 28.6 | 10.4 | | | | | |
| | 9 | 52.2 | 32.3 | 8.5 | | | | | |

TABLE I—*Continued.*

| Dog No. | Hrs. after feeding. | Blood analysis. | | | Urine analysis. | | | | Remarks. |
|---|---|---|---|---|---|---|---|---|---|
| | | Non-protein N. | Urea N. | $NH_2$ N | Total N. | Urea N. | $NH_2$ N | $NH_2$ N | |
| | | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg.* | *mg.* | *mg.* | *mg.* | |
| | 0 | 48.8 | 22.4 | 8.8 | | | | | Fasted again. Lost 24 per cent. 212 gm. Dryco by tube (68 gm. protein). |
| | 1.5 | 52.2 | 25.2 | 8.6 | | | | | |
| 8 | 3 | 54.6 | 25.6 | 8.9 | 798 | 636 | 17 | | |
| | 5 | 55.0 | 27.8 | 9.0 | 675 | 599 | 12 | | |
| | 9 | 56.1 | 30.3 | 9.3 | 1350 | 1198 | 24 | | |
| | 0 | 30.6 | 11.8 | 9.2 | | | | | Well nourished. 100 gm. Dryco by tube (30 gm. protein). |
| | 1.5 | 31.4 | 13.6 | 7.3 | | | | | |
| | 3 | 34.6 | ? | 8.7 | 707 | 595 | 18.5 | 3 | |
| | 5 | 34.3 | 14.6 | 7.9 | 575 | 490 | 11.5 | 5 | |
| | 9 | 29.3 | 12.4 | 7.6 | 535 | 480 | 26.5 | 10.5 | |
| | 0 | 35.5 | 17.7 | 6.7 | | | | | Fasted, losing 25 per cent. 100 gm. Dryco as before. |
| | 1.5 | 40.3 | 20.3 | 6.7 | | | | | |
| 9 | 3 | 37.3 | 18.9 | 6.4 | 846 | 732 | 25 | 5 | |
| | 5 | 39.7 | 20.8 | 7.7 | 565 | 532 | 21 | 3.6 | |
| | 9 | 35.2 | 19.7 | 7.7 | 1316 | 1164 | 56 | 11 | |
| | 0 | 32.3 | 11.9 | 8.3 | | | | | Fed regularly once more. Original weight not yet restored. 100 gm. Dryco. |
| | 1.5 | 35.7 | 12.7 | 8.9 | | | | | |
| | 3 | 32.6 | 12.1 | 8.9 | 798 | 623 | 38 | 17 | |
| | 5 | 31.9 | 14.3 | 8.9 | 1214 | 1001 | 89 | 25 | |
| | 9 | 26.7 | 13.6 | 8.1 | 1016 | 806 | 33 | 19 | |
| | 0 | 34.0 | 11.6 | 8.6 | | | | | Well nourished. 218 gm. Dryco by tube (70 gm. protein). |
| | 1.5 | 36.8 | ? | 8.6 | | | | | |
| | 3 | 36.5 | 12.9 | 8.9 | 1124 | 1018 | 8 | | |
| | 5 | 40.5 | 15.9 | 9.8 | 1140 | 990 | 10 | | |
| | 9 | 37.7 | 13.8 | 10.4 | 1875 | 1711 | 67 | | |
| 10 | | | | | | | | | |
| | 0 | 31.9 | 9.8 | 7.6 | | | | | Fasted, losing 25 per cent. 218 gm. Dryco as before. |
| | 1.5 | 36.0 | 16.8 | 8.0 | | | | | |
| | 3 | 42.5 | 16.2 | 7.8 | 429 | 375 | 16 | | |
| | 5 | 44.8 | 16.5 | 8.1 | 474 | 418 | 8 | | |
| | 9 | 36.0 | 17.3 | 10.5 | 1171 | 1045 | 20 | | |

TABLE I—*Continued.*

| Dog No. | Hrs. after feeding. | Blood analysis. | | | Urine analysis. | | | | Remarks. |
|---|---|---|---|---|---|---|---|---|---|
| | | Non-protein N | Urea N. | $NH_3$ N | Total N. | Urea N. | $NH_3$ N | $NH_2$ N | |
| | | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg.* | *mg.* | *mg.* | *mg.* | |
| | 0 | 29.0 | 9.3 | 7.7 | | | | | Well nourished. 200 gm. Dryco by tube. |
| | 1 | 34.4 | 11.9 | 8.9 | | | | | |
| | 2 | 34.8 | 12.9 | 8.3 | | | | | |
| | 4 | 37.2 | 13.2 | 7.8 | | | | | |
| | 7 | 35.6 | 11.9 | 8.5 | | | | | |
| 11 | | | | | | | | | |
| | 0 | 31.2 | 9.1 | 8.1 | | | | | 200 gm. Dryco as before. |
| | 1 | 40.0 | 11.0 | 8.5 | | | | | |
| | 2 | 45.3 | 11.8 | 8.6 | | | | | |
| | 4 | 46.4 | 13.5 | 9.5 | | | | | |
| | 7 | | | | | | | | Vomited. |
| | 0 | 37.0 | 17.5 | 8.7 | | | | | Well nourished. 100 gm. Dryco by tube. |
| | 1 | 39.7 | 19.0 | 9.6 | | | | | |
| | 2 | 40.1 | 22.0 | 9.8 | | | | | |
| | 4 | 45.5 | 27.5 | 9.1 | | | | | |
| | 7 | 44.5 | 27.1 | 9.5 | | | | | |
| | 0 | 36.8 | 19.4 | 8.4 | | | | | Fasted, losing 20 per cent. Fast broken previous day. 100 gm. Dryco. |
| | 1 | 39.0 | 20.0 | 7.9 | | | | | |
| | 2 | 39.7 | 22.9 | 7.4 | | | | | |
| | 4 | 41.2 | 23.0 | 8.5 | | | | | |
| | 7 | 44.5 | 26.9 | 11.0 | | | | | |
| 12 | | | | | | | | | |
| | 0 | 33.7 | 14.9 | 8.5 | | | | | 4th day after breaking fast. 100 gm. Dryco. |
| | 1 | 31.9 | 18.0 | 8.0 | | | | | |
| | 2 | 32.1 | 17.3 | 7.0 | | | | | |
| | 4 | 32.5 | 19.2 | 7.1 | | | | | |
| | 7 | 33.6 | 18.1 | 7.0 | | | | | |
| | 0 | 32.0 | 11.4 | 8.1 | | | | | 8 days after breaking fast. 100 gm. Dryco. |
| | 1 | 42.0 | 25.0 | 8.1 | | | | | |
| | 2 | 39.2 | 21.3 | 8.2 | | | | | |
| | 4 | 39.5 | 21.0 | 9.2 | | | | | |
| | 7 | 40.5 | 20.6 | 10.3 | | | | | |

TABLE I—*Concluded.*

| Dog No. | Hrs. after feeding. | Blood analysis. | | | Urine analysis. | | | | Remarks. |
|---|---|---|---|---|---|---|---|---|---|
| | | Non-protein N. | Urea N. | $NH_3$ N | Total N. | Urea N. | $NH_3$ N | $NH_3$ N | |
| | | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg.* | *mg.* | *mg.* | *mg.* | |
| | 0 | 36.6 | 16.5 | 8.2 | | | | | Well nourished. Mixture of lactose and starch by tube. |
| | 1 | 33.3 | 17.6 | 8.4 | | | | | |
| | 2 | 36.9 | 17.3 | 8.4 | | | | | |
| | 4 | | | | | | | | |
| | 7 | 36.1 | 17.0 | 8.2 | | | | | |
| 13 | | | | | | | | | |
| | 0 | 39.4 | 17.9 | 8.8 | | | | | Fasted, losing 14 per cent. Same experiment as before, following 6 days of refeeding. |
| | 1 | 35.1 | 18.4 | 7.8 | | | | | |
| | 2 | 33.7 | 17.2 | 7.5 | | | | | |
| | 4 | 33.8 | 17.0 | 7.3 | | | | | |
| | 7 | 30.9 | 16.8 | 6.6 | | | | | |

and in the extent of the urea nitrogen rise in the prefasting and postfasting conditions is very striking. A study of the nitrogenous products eliminated in the urine during these experiments shows that this difference in the blood urea concentration could *not* be attributed to a retention, since the urea excretion of this dog during the postfasting experiment even exceeded somewhat the amount of urea eliminated for the same length of time when the dog was in a well nourished state (3591 and 2802 mg. urea N respectively). The total N excretion was not so different in the two experiments: 4017 and 3833 mg.

In the experiments with Dog 8 we have two parallel sets of tests, one with meat and one with Dryco powder. Each set of experiments was performed with the dog in the prefasting and in the postfasting condition. In the first two experiments the amino acid N of the blood fell below the original level 1½ hours after feeding. The blood sample 5 hours after feeding shows the first increase above the 0 level, and continues increasing as the digestive process continued for the next 4 hours. The urea N curve, in the meantime, behaves quite differently. In the pre-

fasting experiment the urea N increased slightly by the end of 3 hours after feeding (2 mg.), its subsequent decline coinciding with the rise in amino acid N. The concomitant rise in amino acid N and fall in urea N is not uncommon in the more advanced stages of digestion and absorption. During the postfasting experiment, on the other hand, the urea N curve rises continuously and rapidly, while the amino acid N does not begin to increase until the urea N has already increased 25 per cent. From this point both the amino acid N and the urea N curves rise rapidly.

In the next test with this dog Dryco was fed by stomach tube. Although the dog had by this time already regained the weight lost in the previous fast of 16 days, and had been liberally fed during the interval between the two tests, physiologically it was evidently not yet restored to the normal condition. The blood changes found during digestion are still essentially characteristic for a postfasting condition. The urea N curve rises rapidly and continuously following the introduction into the stomach of a definite amount of Dryco. By the end of 9 hours the blood urea concentration has increased 50 per cent, while the amino acid N rises slowly during the first 3 hours and at the close of the 9th hour has once more declined to the original level. The dog was again fasted until the weight had diminished about 25 per cent, and the feeding experiment was repeated using the same amount of Dryco as before. In this test the amino acid N remained once more practically unaltered for the first 3 hours after feeding, increasing inconsiderably during the next 6 hours. The urea N of the blood, however, rises steadily from the start, increasing in 9 hours up to 30.3 mg. from the original level of 22.4 mg. The results of the two sets of experiments are, therefore, very similar. Unfortunately, the urine for the first experiment was lost, but the urine analyses during the second experiment indicate that the striking changes in the blood urea N cannot be ascribed to retention, as was also demonstrated in the previous test.

Dog 9 was tested both before and immediately after fasting, and once more when it was fed up nearly to its original weight. The food used in these tests was the same quantitatively and qualitatively (Dryco). In the prefasting experiment the amino acid N of the blood diminished during the process of digestion.

The curve is very irregular but even 9 hours after feeding it remained below the original level (0 time). The blood urea N, on the contrary, shows an increase already in the first blood sample. In the next two experiments the amino acid N of the blood either changes very insignificantly or shows an increase only after the blood urea N had already reached a maximum value. Similarly, in the case of Dog 10, the amino acid N of the blood begins to increase when the urea N curve has already commenced to decline or is still rising. This experiment brings out once more the striking fact of the large rise in the blood urea N coincident with digestion and absorption of food in a postfasting condition.

The experiments with Dog 12 are especially interesting because they show the progressive change in the postfasting condition, the animal having been tested 2, 4, and 8 days after breaking the fast. In the prefasting test, following a small initial rise in the blood amino acid N 1 hour after passing food into the stomach, there is practically no further change for the next 6 hours, while the urea N increases from 17.5 to 27.5 mg. in the first 4 hours of the experiment. The tests were then repeated when the dog had fasted for several days and had lost 20 per cent in weight. In the experiment performed on the 2nd day after breaking the fast the blood urea N behaved practically as it did before the fast, while the amino acid N actually diminished during the first 2 hours, and rose above the original level only in the sample obtained 7 hours after feeding. The urea N has been increasing gradually and continuously during the entire experiment. Similarly, 4 days after breaking the fast essentially the same changes were observed in the blood, except that this time there was no rise in the amino acid N even in the advanced stage. Likewise, 8 days after the fast was broken the amino acid N of the blood remained unchanged for 2 hours after feeding, but increased rapidly between the 2nd and 7th hours from 8.2 to 10.3 mg. In the meantime the blood urea N increased from 11.4 to 25 mg. within the 1st hour after feeding. In this experiment the rise in amino acid N again coincided with the fall in urea N, a fact already noted in the advanced stages of digestion and absorption in previous tests.

The blood changes here described are incidental to the absorp-

tion from the digestive tract of nitrogenous products and not to a stimulation of the physiological activity of the digestive glands. This is shown by the control experiment on Dog 13 fed on a mixture of lactose and starch. Except for the difference in food the rest of the experimental procedure was exactly as in the previous experiments, and the dog was tested both in the well nourished and in the postfasting condition (after a loss of 14 per cent in weight).

## DISCUSSION.

The experimental results presented above furnish, therefore, evidence that during the absorption of protein digestion products the urea N of the systemic blood frequently increases *before* the amino acid N. *This can be seen particularly well in animals tested a short time after breaking a preliminary protracted fast.* Whereas this fact, in accordance with the theoretical expectation postulated by Folin and Berglund, can be regarded as definite proof that the liver is the predominant organ for urea formation, it may be pointed out that the few instances where both the urea and the amino acid N show an initial increase probably indicate nothing more than a flooding of the system with amino acids beyond its capacity either to deaminize or convert them to urea within the given time. *It should also be noted that not infrequently a fall in the amino acid N of the blood has been found in the early stages of the digestive process.* We, therefore, believe that our experiments furnish proof of the urea-forming function of the liver in the intact animal, a fact already demonstrated by Bollman, Mann, and Magath on hepatectomized animals.

To test further the correctness of this conclusion of the urea-forming function of the liver we experimented also with two dogs with a true Eck fistula.[2] The animals made good recovery from the operation and were gaining weight on a diet of milk, corn syrup, and bread. Subsequent postmortem examination showed that the operation was perfect and that the portal circulation had been completely cut off. The liver was extensively

[2] The two Eck fistula dogs were sent to me by Dr. Frank C. Mann of the Mayo Foundation in whose laboratory the operation was performed. I wish to acknowledge my appreciation as well as my indebtedness to Dr. Mann for this.

atrophied at the time of the autopsy,[3] about 2 months after the operation was performed, and it is reasonable to assume that at the time the experiments were made, 1 month after the operation, there was also considerable atrophy. Many of the data obtained with these two dogs baffle every attempt of interpretation, but the blood picture of these Eck fistula dogs is worth pointing out here. The blood was taken as usual directly from the left heart and 24 hours after feeding. *The urea concentration of their blood has been found to be exceptionally low,* 2.9 to 6.4 mg. per 100 cc. in Dogs 14 and 15, *whereas the non-protein N was only somewhat lower than it is generally found in normal dogs and the amino acid N was entirely normal.* It is a familiar fact, of course, that the organism possesses more liver tissue than is required physiologically, and therefore the liver can probably undergo considerable atrophy without revealing any functional disturbance. We suggest tentatively that the extremely low urea N values of the blood of our Eck fistula dogs may not unreasonably be regarded as pointing to the importance of the liver in the urea-forming process in the organism.

Although it might have been anticipated that in Eck fistula dogs there would be a striking rise in the amino acid N without a corresponding increase in the urea N during digestion and absorption of protein food, this *a priori* expectation is realized only partially and in a modified form. The blood urea N changes after the ingestion of food were similar to those already described for the non-operated dogs. The amino acid N, in all the six experiments with the Eck fistula dogs, increased, and this increase was always found the 1st hour after feeding. It will be recalled that the amino acid N curve of the non-operated dogs did not behave in such uniform manner, frequently remaining unchanged or even diminishing in the early hours of digestion. Another important difference between the normal and the Eck fistula dogs, as far as the amino acid N of the blood is concerned, is the fact that in the latter much higher values (up to 13 to 14 mg.) have been found than were ever observed in non-operated animals. Von Falkenhausen (6) reports that upon feeding 20

[3] I am greatly obliged to my colleague Dr. H. E. Eggers, Professor of Pathology, for his kind cooperation in performing the autopsy and studying the histological preparations from the various tissues.

gm. of a mixture of amino acids to patients with *no* liver involvement the amino acid N of the blood increases at the end of 60 minutes 32 to 47 per cent, but in patients suffering from various liver affections (without icterus!) the increase varies from 47 to 86 per cent. It must be emphasized, however, that experiments like those of von Falkenhausen have a common defect in that the organism is subjected to a maximum strain. Nevertheless, they would seem to indicate a relationship between the functional activity of the liver and the rate of disposal of circulating amino acids. A more remarkable condition bearing upon this problem is presented by the hibernating animal. It is a well established fact that in hibernation the glandular organs are in a state of distinct hypofunction. It has been observed that in the hibernating animal a very singular thing occurs, namely, that the urea, which is generally the chief nitrogenous component of the urine during the waking period, assumes a secondary importance during hibernation, while the amino acids become the principal single nitrogenous waste product eliminated through the urine (7). This fact, though it cannot be regarded as a direct proof, is nevertheless very suggestive of the probability of a connection between liver hypofunction and a diminished excretion of urea. Finally, the interesting observation of McCance (8) that ammonia is formed in the autolysis of all mammalian organs while urea is formed only in autolysates of liver, kidney, or spleen *but not of muscle tissue*, may be mentioned as adding light to an obscure problem.

In conclusion it must be pointed out that what we generally designate as "urea formation" may in reality not represent a single process at all. It is not improbable that the deamination of the amino acids and the synthesis of urea may be independent processes. At any rate, the experiments of von Falkenhausen and Siwon (9) on hepatectomized geese, though they stand in need of verification, seem to indicate that such a distinction is really necessary, and that the predominant rôle of the liver may be limited to the process of urea synthesis proper. It is possible that a closer approach towards a solution of this problem will be attained from this angle.

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# A COLORIMETRIC METHOD FOR THE DETERMINATION OF THE pH OF CEREBROSPINAL FLUID.*

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Recently the pH of the blood has been exhaustively studied but that of the cerebrospinal fluid has received little attention. Because the composition of spinal fluid is less complex, the determination of pH and interpretation of the results should be simpler than for blood. Cerebrospinal fluid is not only free from hemoglobin but also contains so little protein as to be, for practical purposes, protein-free. Is spinal fluid a transudate or a secretion? Is its relation to the blood such that it fulfills the laws of the Donnan equilibrium? The simultaneous determination of the reaction of blood and of spinal fluid give data applicable to such questions.

In the course of an investigation on the ionization of calcium it became evident that test-tube experiments, involving a simple water system, could not be applied directly to the body fluids in which the protein effect is at present not subject to quantitation. Therefore, an effort was made to apply such consideration to a body fluid free from protein, namely, cerebrospinal fluid. One of the necessary determinations is an accurate estimation of pH.

## HISTORICAL.

Foa (1), in 1906, was the first to use the electrometric method for measuring pH of spinal fluid. He found a pH of 7.22 for a dog. In 1911 Polányi (2), working in Tangl's laboratory, found values approximating pH 10. Bisgaard (3), reports values determined for him in the Carlsberg laboratory by

* Read at the Washington meeting, December, 1924, of the American Society of Biological Chemists.

Paltiszch. After passing $H_2$ through spinal fluid all night, he found the reaction to be more alkaline than pH 9.0. He also used Sörensen's buffer standards and the colorimetric method on fresh fluid dropping directly from the needle and found the fluid acid to phenolphthalein. In 1916, using phenol red as an indicator and reading the pH values colorimetrically either directly or on the dialysate through collodion membranes according to the method of Levy, Rowntree, and Marriott, Hurwitz and Tranter (4) obtained values of pH 8.11 on the dialysate and 8.26 directly; and Weston (5) found pH 7.9 to 8.3 on the dialysate. Levinson (6), employing both the gas chain and phenol red in open and stoppered tubes and at various intervals after lumbar puncture, obtained values of pH 7.7 to 7.9 on fresh material. Felton, Hussey, and Bayne-Jones (7), who used phenol red, record the value of spinal fluid immediately after removal as 7.4 to 7.6. Levinson and Felton, Hussey, and Bayne-Jones came to the conclusion that allowing spinal fluid to stand unprotected had permitted the escape of $CO_2$, hence, previous studies had given values much too alkaline. Ylppö (8), used the electrometric method with the refinements of Hasselbalch and Michaelis, but recognized the error in his determinations through loss of $CO_2$; Ylppö obtained values of pH 8.4. Milroy (9) made an electrometric determination at 37.5°C. of a single spinal fluid and found a value of pH 7.24 at 29.3 mm. $CO_2$ tension and concluded that spinal fluid approximates a solution of 0.02 N $NaHCO_3$ in 0.18 N NaCl. McClendon (10) introduced a method of titrating the alkaline reserve and then calculating the pH. Parsons and Shearer (11) studied three cases by electrometric method at various $CO_2$ tensions on two specimens obtained without contact with air. They calculated that under body conditions the pH was 7.32 and 7.12 at $CO_2$ tensions of 48 mm. and 64 mm. respectively. In a second paper, Shearer and Parsons (12) report pH values of 7.4, 7.4, and 7.3 for three normal individuals. The colorimetric method was employed and all readings were made at room temperature. Klothilde Meier (13), at approximately the same time, made a similar colorimetric pH measurement on fluid received under albolene without contact with air. She also plotted absorption curves and thus calculated the tension of $CO_2$ in the body. On two normal cases she obtained pH 7.38 and 7.33 and, by calculation, in the second case pH 7.35 under a tension of 39 mm. $CO_2$. This is the first time that values so closely approaching those of normal blood were obtained. For fluids from pathological cases Meier found extreme variations of pH 7.11 to 7.58. Using Michaelis' bufferless standards of M nitrophenol, Brock (14) measured the pH of spinal fluid, obtained under paraffin oil at room temperature, and obtained values of 7.50 to 7.55 with a $CO_2$ tension of 24 to 27 mm. He tested his standards against Sörensen's phosphate buffer standards and found his values too alkaline by 0.025 pH. He was not unmindful of the difference of temperature and determined the pH at 38°C. but was unable to detect differences of more than 0.02 pH to the acid side. After saturation with expired air at 40 mm. tension he obtained values of pH 7.28 to 7.30. Behrendt (15) and Waltner (16) (1924) used Brock's method and confirmed his results.

*Principle of Method.*

The earlier work does not take cognizance of the importance of the $CO_2$ tension. Loss of $CO_2$ resulted in a pH value much too alkaline. At first, values of pH 9.0 to 10.0 were found, but with increasing precaution to prevent the escape of $CO_2$, the values have gradually approached that of the blood.

The principles of the method that we have used are, first, to prevent loss of $CO_2$, and second, to determine the pH colorimetrically at body temperature, 38°C.

*1. Loss of $CO_2$.*—The investigations of Van Slyke and his collaborators have shown that the precautions necessary for the handling of blood or plasma are not adequate in dealing with bicarbonate solutions. Spinal fluid approximates a solution of sodium bicarbonate in physiological saline. Therefore, we attempted to fulfill the following conditions: first, the spinal fluid must never come in contact with air; second, removal under mineral oil should be replaced by collection over mercury; third, all transfer should be avoided. By following the principle of the Van Slyke technique the apparatus shown in Fig. 1 was devised.

The apparatus was made from one of the tubes used for the pH standards so as to insure uniform size, color, thickness, and material. To one end a three-way stop-cock and to the other a straight tube is fused. These are suitably calibrated, the narrow portion between the tube and stop-cock at $_1{}^1{}_0$ cc. and the remainder at 1 cc. marks. A rubber tube with pinch-cock and a small mercury leveling bulb complete the apparatus.[1]

*2. Colorimetric Comparison at Body Temperature.*—The necessary data for the temperature factors have been furnished in the able paper of Hastings and Sendroy (17). The essential point is that a solution of phosphates prepared according to the technique of either Sörensen or Clark and containing phenol red apparently becomes more alkaline when heated. If a standard at pH 7.20, containing phenol red, is heated to 38° and compared with an identical standard at 20°, the warmer tube appears redder. In order to match the standard at 38° which formerly was pH 7.20 the standard used must be at pH 7.28 at 20°.

[1] The apparatus and comparison tubes were supplied by the Empire Laboratory Supply Company, New York City.

The change is due to two factors. First, the phosphates really become more acid by 0.03 pH; in other words, the $pK_1$ of the phosphates becomes less or the acid becomes stronger by 0.03; second, the indicator, which is also a weak acid, has become stronger and binds more base. Therefore, more of the indicator appears in its alkaline or red form. If both the solution and the

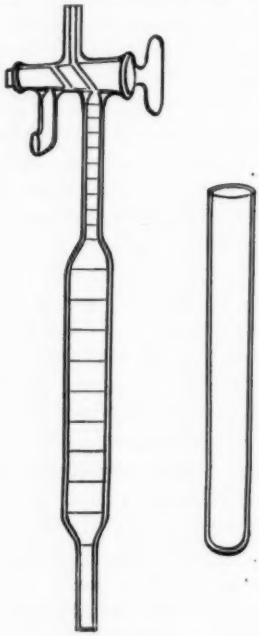

FIG. 1.

standard are observed at 38° the effect due to the action of the increased temperature on the dye is equal in the two tubes and hence requires no correction. The only alteration is that of the actual pH value of the standard. By calling a standard which is pH 7.20 at 20°, pH 7.17 at 38°, solutions may be compared with the Sörensen standards when both are at 38°.

The method devised by Hastings and Sendroy employs bicolorimetric standards suitable for 38°. These are prepared by dividing

a constant quantity of indicator into varying amounts. One fraction is put into a tube containing 0.001 N HCl; the other tube contains 0.01 N NaOH. These two tubes replace the single buffer standard. The color which in the buffered standards depends upon mixtures of acid and alkali salts in the same tube is here produced by the use of two. This method has three advantages; the standards are easier to make, are more permanent, and are independent of the effect of temperature. Only the apparatus containing the spinal fluid plus the indicator solution need be heated to 38°. The solutions and standards are then compared in a suitable colorimeter such as described in principle by Bjerrum or Gillespie. Since the protein in spinal fluid is absent, or present in small amounts, no protein error results. The effect on the indicator due to the salt concentration has been studied by Hastings. This is constant and is 0.02 of a pH.

*Procedure.*

Measure into the apparatus as many tenths of cc. of Hastings' indicator solution (0.0075 per cent phenol red), as of cc. of spinal fluid which it is expected to use. Connect the upper capillary tube directly to the lumbar puncture needle by means of a rubber tube bearing a glass Luer adapter previously sterilized, observing sterile precautions the while. Allow the fluid to escape under its own pressure through the side of the three-way stop-cock which has been turned so as not to be connected with the apparatus. When all air has been removed from the capillary tube, allow the spinal fluid to enter the apparatus by turning the stop-cock and holding the bulb in such a position that its mercury level is at or very slightly below that in the sampling tube. The flow of spinal fluid is stopped when the desired amount has entered. Place a pinch-cock at the lower end of the apparatus. Immerse in a water bath at 38° for 5 minutes and compare with the bicolorimetric standards.

*Results.*

In Table I comparisons of the blood pH and spinal fluid pH are given. These results are selected from over 50 determinations. The one specimen from Dog 4 whose blood showed a pH 7.20 is included because it is our most discordant determination. It

shows possible but not probable error. In cases with infection of the meninges the spinal fluid is more acid than the blood. It will be seen that normal spinal fluid pH and blood plasma pH are the same within the sum of the errors of the two methods. The measurement of the pH of spinal fluid has equalled in accuracy that of the blood, and its relation to the equilibrium of the body can therefore be evaluated.

TABLE I.

*Comparison of the pH of Blood and Cerebrospinal Fluid.*

| | Blood (arterial). | Spinal fluid. |
|---|---|---|
| | *pH* | *pH* |
| Dog 1 | 7.33 | 7.37 |
| " 2 | 7.32 | 7.39 |
| " 3 | 7.37 | 7.35 |
| " 4 | 7.20 (Venous.) | 7.37 |
| Scarlet fever (convalescent) | 7.35 | 7.35 |
| Normal | 7.32 | 7.32 |
| " | 7.39 | 7.36 |
| Syphilis | 7.34 | 7.34 |
| Encephalitis | 7.38 | 7.32 |
| " | 7.42 | 7.29 |
| " | 7.35 | 7.28 |
| " | 7.36 | 7.28 |
| Acidosis | 7.17 | 7.26 |
| Meningitis | 7.38 | 7.30 |
| " | 7.47 | 7.21 |
| " | 7.47 | 7.26 |
| " | 7.42 | 7.27 |
| " | 7.37 | 7.26 |

DISCUSSION.

To analyze the results even of the latest and best investigations of the pH of the spinal fluid is difficult because experimental errors are not considered in the calculations. Parsons and Shearer place the normal pH at 7.32 and 7.12 with $CO_2$ tensions of 48 and 64 mm., Meier at pH 7.33 and 7.38 with $CO_2$ tensions of 30 and 39 mm., Waltner and Brock at pH 7.50 and 7.55 with $CO_2$ tensions of 24 and 27 mm. The most alkaline values are associated with the lowest $CO_2$ tension and *vice versa*. The question

of absorption curve of spinal fluid offers many difficulties involving the temperature, the absorption coefficient, the total salt concentration, and the values of $pK_1$ used. The values given are consistent, but it is obvious that in three sets of experiments, all on normal individuals, the spinal fluid cannot be 50 per cent above that of the alveolar air in one case, equal to it in another, and 50 per cent below it in a third. There must be an error either in the $CO_2$ tension or in the pH. If the spinal fluid is in equilibrium with the blood a constant relationship must exist between the pH of both. A difference of 10 mm. in $CO_2$ tension causes a change in reaction of roughly 0.10 pH.

The difficulty of applying electrometric method to dilute carbonate solutions without other buffer under varying $CO_2$ tensions is great for several reasons. The absorption curve is so flat that small difference in tension causes large difference in pH and but small difference in the total $CO_2$. Second, the equilibrium is established so slowly as to make electrometric determinations either with solutions saturated with known mixtures or by refill method extremely difficult. If the colorimetric principle can be applied correctly, it should give more accurate and more nearly constant results. The temperature coefficient of the solutions, as worked out by Hastings and Sendroy, is important. Our tests have likewise shown a difference of about 0.06 pH between the measurements at 20° and 38°. Brock was probably in error because Austin and his coworkers have shown that $CO_2$ solutions, when heated, cannot be protected against loss of $CO_2$, unless paraffin and not paraffin oil is used. It seems much easier and simpler to heat the solution to the desired temperature over mercury.

It is interesting that normally the acidity of the blood and spinal fluid, simultaneously determined, should be so nearly equal. As the error of each reading is 0.02 pH, the total difference should be not 0.04 pH but 0.02 pH. If the determinations are made at the same time, the two tubes can be compared directly. The advance in accuracy is due to improved technique with regard to (1) prevention of loss of $CO_2$; (2) determination of pH at body temperature with improved colorimetric standards.

SUMMARY.

A comparison of blood and cerebrospinal fluid shows that the two normally have the same pH, 7.35 to 7.40 ± 0.02.

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# THE COLORIMETRIC DETERMINATION OF PHOSPHORUS.

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The Bell and Doisy method[1] for the determination of phosphate, based on the reduction of phosphomolybdic acid by hydroquinone to a substance which is blue in the presence of sulfite, had one weakness, the rapid and irregular fading of the color in alkaline solution. By omitting the use of alkali, Briggs[2, 3] has succeeded in eliminating this particular difficulty, but others, in some respects more serious, have at the same time been introduced. Briggs has suggested so many variations of his own method that no statement that could be made about any one of them would apply precisely to them all. We shall confine ourselves for the most part to the two (one for blood and the other for urine) described in his first paper on the subject.[2]

In the urine method, where ordinarily the greatest accuracy would be needed, the conditions during the synthesis of phosphomolybdic acid and its subsequent reduction by hydroquinone are distinctly different from those proposed by Bell and Doisy; chiefly, the concentration of hydroquinone called for in Briggs' directions is only $\frac{1}{50}$ as high. To counterbalance this to some extent, Briggs prolongs the reaction time from 5 to 30 minutes, but the color produced even then is certainly not more than four-fifths of the maximum. The reduction of phosphomolybdic acid by hydroquinone must be a reaction of at least the second order. To get proportionate readings with a bimolecular color reaction, one condition must absolutely be fulfilled—the reagent must be added in such large excess that the reaction runs an apparently unimolecular course. In Briggs' urine method, where this is not

[1] Bell, R. D., and Doisy, E. A., *J. Biol. Chem.*, 1920, xliv, 55.
[2] Briggs, A. P., *J. Biol. Chem.*, 1922, liii, 13.
[3] Briggs, A. P., *J. Biol. Chem.*, 1924, lix, 255.

the case, the deviation from proportionality with a solution one and two-thirds times the strength of the standard is as much as 3 per cent.

In the method which he first proposed for blood, Briggs did increase the speed of the reaction by using more of the reagents in proportion. Thus, he recommends 1 cc. of 0.5 per cent hydroquinone whether the final volume is to be 100 cc. (as with urine) or 10 cc. (as it ordinarily would be in the analysis of blood). The concentration of hydroquinone is hence ten times as high in the latter case; and the concentration of the acid molybdate reagent is similarly increased four times. Other things being equal, the proportionality should thereby be improved, but actually it is made distinctly worse. The reason is that the molybdic acid and hydroquinone, in the absence of phosphate, then give a brownish yellow color which is partly, though by no means wholly, discharged by sulfite.[4] The blank so introduced has a colorimetric value equal to more than 10 per cent of the blood standard, and unless the standard and unknown are nearly equal the tints are not at all alike. Accurate matching under such conditions is very difficult, if not impossible. According to Briggs, this method gives exact results with solutions one and two-thirds times as strong as the standard, and also with less than half the standard quantity of phosphate; but in our experience the error in the former case is about 4 per cent, and in the latter fully 10 per cent.[5]

[4] In the Bell and Doisy method this color is destroyed by adding alkali, and hence does not interfere.

[5] More recently,[3] in connection with the analysis of blood, Briggs proposes to intensify the color by means of two modifications, neither of which accomplishes that object. Since an excess of acid diminishes the color he aims, by using less of the acid molybdate reagent, to make it stronger,—but in this respect the two constituents of the reagent (molybdate and sulfuric acid) practically balance each other, and actually there is nothing gained. Postponing the addition of the sulfite until the reduction is well under way (his second alteration) does accelerate the development of color in the early stages, but at the end of 30 minutes it is no deeper than when sulfite is added at the start. The somewhat greater color which Briggs now gets, as compared with his original blood method, is the result of two quite different changes, on which he makes no comment. That is to say, he doubled the amount of hydroquinone, and likewise increased the standard from 0.025 to 0.03 mg. of phosphorus. The blank is not appreciably different in the modified procedure.

Since small amounts of hydroquinone reduce too slowly, and large amounts give a color with molybdic acid itself, it is clear that a really satisfactory method based on this principle calls for a reducing agent which is much more rapid. Such an agent, to be thoroughly acceptable, must bring about the practically complete reduction of phosphomolybdic acid within a reasonable period when added in a concentration too low to give a blank.

In our attempts to solve this problem, we have had in mind a method applicable to material of widely varying nature, and not merely the demands of ordinary blood and urine analysis. There the conditions are comparatively simple—besides the anticoagulant, the only interfering substances known to be important are the trichloroacetic acid with which the protein is precipitated and the sulfuric acid used for the digestion in total phosphorus determinations. While these things, as Martland and Robison[6] have found, are not so harmless in the Briggs method as was at first supposed, minor alterations in technique suffice to make them so in so far as blood filtrates are concerned.[7]

The determination of total phosphorus in the presence of much organic matter is a somewhat different proposition, for ashing with sulfuric acid (aided by nitric acid or hydrogen peroxide) has its limitations. The quantity of sulfuric acid used must not be too large, otherwise the reduction of phosphomolybdic acid will be retarded; and it must not be too small, or there will be danger of loss of phosphate (variously ascribed to volatilization,[8] to the formation of pyro- or metaphosphoric acid,[1] and to combination with the glass).[6, 9]

Organic matter which gives rise to picric acid when digested by the so called Neumann method offers special difficulties—a completely colorless solution is then sometimes impossible to get without danger of overheating, and consequent loss of phosphate. In cases of this kind, and in fact when dealing with very refractory material of any sort, dry ashing is the only way left open. The most widely used reagent for this purpose—a mixture of sodium

[6] Martland, M., and Robison, R., *Biochem. J.*, 1924, xviii, 765.

[7] With respect to trichloroacetic acid, this is not always true. The point will be considered later.

[8] Baumann, E. J., *J. Biol. Chem.*, 1924, lix, 667.

[9] Whitehorn, J. C., *J. Biol. Chem.*, 1924–25, lxii, 133.

carbonate and potassium nitrate—causes hopeless complications in the subsequent steps of Briggs' method. In order to provide the proper acidity for the color reaction, the carbonate must first of course be neutralized. The most convenient, and practically the only feasible way to do this is by evaporation to dryness with a small excess of hydrochloric acid. The residue is then chiefly sodium chloride, with some nitrate, and analysis of this mixture with any accuracy (by Briggs' method) is precluded by the fact that chloride and nitrate both intensify the phosphate color, while nitrite (if any should be present) would have the opposite effect. Even with material that can be ashed with carbonate alone, erroneous results will be inevitable if the amount of carbonate exceeds about 0.3 gm.

Another source of error to be reckoned with, when the organic matter has been destroyed by alkali, is silicate. In 30 minutes, by Briggs' method, 1 mg. of Si gives as much color as 0.4 mg. of P. Some plant and animal tissues contain considerable silicate, and a rapid colorimetric phosphate method not affected by this substance might be found useful also in other fields than biochemistry.

In connection with the determination of total phosphorus in such materials as blood, regardless of the method of digestion, it is worthy of note that the reduction of phosphomolybdic acid is markedly retarded by ferric salts. Blood generally contains more iron than phosphorus; in some species four or five times as much—enough to introduce a serious error.

We have listed, in Table I, data which will serve to show, in a general way, how the development of color by hydroquinone is affected by the interfering substances which have been mentioned. Included there as well are the results of some observations with ammonium sulfate, which Rimington[10] has already shown to modify the color. His data are presented only in the form of graphs, and from them it is impossible to judge the safe minimum concentration of this salt. Rimington's main concern was with the diminution of color intensity in strong ammonium sulfate solutions. The increase in color in more dilute solutions, which he passes over with slight comment, seems to us at least equally important.

[10] Rimington, C., *Biochem. J.*, 1924, xviii, 1297.

If the departure from exact proportionality (amounting to a few per cent) were the only drawback, the Briggs method might still be made accurate enough by the use of appropriate corrections. But no such correction could be relied upon if the color does not

TABLE I.

*Effect of Various Substances on Color Production in Briggs' Urine Method.*

Volume, 100 cc. 0.5 mg. P. Time, 30 min. Standard set at 20 mm.

| Substance added. | Reading. |
|---|---|
| | mm. |
| Sulfuric acid (additional), 0.1 N | 20.3 |
| 0.2 " | 20.5 |
| 0.3 " | 20.9 |
| Sodium chloride, 0.2 M | 19.4 |
| 0.5 " | 18.2 |
| M | 17.0 |
| Potassium nitrate, 0 04 M | 19 6 |
| 0.2 " | 18.2 |
| 0.4 " | 16.9 |
| Sodium nitrite, 0.002 M | 20.2 |
| 0.01 " | 22.0 |
| 0.02 " | Yellow. |
| Ammonium sulfate, 0.05 M | 19.5 |
| 0.25 " | 18.1 |
| 0.5 " | 17.7 |
| Ferric chloride, 1 mg. Fe | 20.4 |
| 5 " " | 22.3 |
| 15 " " | 30.0 |
| Sodium silicate, 0.25 mg. Si | 19.3 |
| 0.5 " " | 18.8 |
| 2.0 " " | 15.1 |

develop at the normal rate. We have given abundant evidence that many things do affect the rate, and many more undoubtedly could be found.

A reducing agent which would give the maximum color in 30 minutes (*i.e.*, at least 18 per cent more than hydroquinone, see

Table I), would help to only a limited extent. There would still be errors to contend with from the presence of substances which retard the color reaction. The first reducing agent that we found, capable of yielding about 20 per cent more color than hydroquinone in half an hour, was 5-aminosaligenin. By this time we had come to appreciate the tremendous advantage to be gained by the use of a reagent which would reduce phosphomolybdic acid (selectively of course) at a rate many times more rapid still. The necessity of waiting 30 minutes would be dispensed with, in the first place. But more important, a reducing agent which would normally give almost the maximum color within 5 minutes, let us say, would provide a means of determining (when working with unknown material) whether or not the color reaction is proceeding at the normal rate and when it is complete. In other words, the presence of any interfering substance would then be automatically revealed by a change in the colorimeter reading from one minute to the next. Finally, a very material increase in reduction rate offers the only hope of getting accurate proportionality of color over a wide range, without waiting several hours.

It is natural to expect that any reducing agent capable of meeting this criterion would itself be more or less unstable. Besides activity, therefore, the keeping qualities of the reagent had to be kept constantly in mind, as well as its solubility and the ease with which it can be prepared and purified. Two substances (2-chloro-4-methylaminophenol and 2, 4-diaminophenol) were found which, although better than anything that had been tried before, were still not entirely satisfactory from every standpoint.[11]

At this juncture we tried an old sample of 1, 2, 6-aminonaphtholsulfonic acid (which was in stock and had been partially purified) with very encouraging results. Impure as it still was, it gave more color than anything that had been tested previously. A pure sample of this acid, made following the directions of Meldola,[12] gave about the same result. It acts so rapidly, in fact,

[11] Several other compounds have been tried. Monochlorohydroquinone and 2,5-dichlorohydroquinone are even slower than the unsubstituted phenol, while *p*-methylaminophenol and 2-chloro-4-aminophenol are not appreciably better. (For samples of a number of these products we are much indebted to Mr. W. G. Christiansen of the Department of Pharmacology.)

[12] Meldola, R., *J. Chem. Soc.*, 1881. xxxix. 47.

that the color intensity obtained by means of hydroquinone in half an hour is exceeded, in the presence of an equimolecular amount of the sulfonic acid, in a fraction of a minute; its activity as a reducing agent for this purpose must be more than 50 times as great.

1, 2, 6-Aminonaphtholsulfonic acid is fairly difficult to make. The preparation of one of its isomers (the 1, 2, 4 acid) has been described in full detail by Folin[13] as a step in making his amino acid reagent; it is also on the market in crude form, and can be purified without much trouble. Inasmuch as the two isomers reduce phosphomolybdic acid with approximately equal speed, the more available one is naturally to be preferred.

The effect of those substances,[14] most likely to be present in the analysis of biological material, which are known to retard the reduction of phosphomolybdic acid by this new reagent or to interfere in any other way, may be seen in Table II. In the experiments recorded there, the standard was in every case the same—0.4 mg. of phosphorus in a volume of 100 cc., containing 0.25 gm. of ammonium molybdate, 10 cc. of 5 N sulfuric acid, 0.6 gm. of sodium bisulfite, and 0.01 gm. of aminonaphtholsulfonic acid. The solution to be read against this standard contained between 0.2 and 1.6 mg. of phosphorus, besides the added foreign substance; the other reagents were the same as in the standard, except in certain special cases (indicated in the table) where a larger or smaller concentration of sulfuric acid was used. Readings were made at frequent intervals, beginning 2 minutes after adding the sulfonic acid, and continuing until there was no further change.

Allowing 5 minutes for the development of the color, or a little longer if the phosphate concentration is more than twice that in the standard, aminonaphtholsulfonic acid will generally give accurate results in the presence of at least ten times the amount of inhibiting material that would be permissible with hydroquinone. Moreover, when the safe limit has been exceeded, the fact will be made evident by a lack of constancy in the reading, and in some cases (ferric salts, ammonium sulfate, nitrite) also by a difference in tint between standard and unknown. We suggest that, when

[13] Folin, O., *J. Biol. Chem.*, 1922, li, 386.

[14] Recrystallized, when necessary to get them phosphate-free.

TABLE II.

*Colorimetric Readings by Proposed Method in Presence of Interfering Substances.*

Standard (0.4 mg. P) set at 20 mm. Volume, 100 cc. Temperature, 25–27°.

| $H_2SO_4$ concentration. | 0.5 N | 0.6 N | 0.7 N | 0.5 N | | | | | | | | | | 0.6 N | 0.3 N |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Substance added.. | 0 | 0 | 0 | M NaCl | 0.4 M $KNO_2$ | $NaNO_2$ 0.005 M | $NaNO_2$ 0.02 M | $(NH_4)_2SO_4$ 0.25 M | $(NH_4)_2SO_4$ 0.5 M | Ferric chloride. 2 mg. Fe. | Ferric chloride. 8 mg. Fe. | Ferric chloride. 15 mg. Fe. | Na silicate. Si:P= 50 | Na silicate. Si:P= 200 | 20 cc. 20 per cent $CCl_3COOH$ + 30 mg. potassium oxalate.‡ |
| Time. | 0.2 mg. phosphorus (theoretical reading, 40.0 mm.). | | | | | | | | | | | | | | |
| *min.* | | | | | | | | | | | | | | | |
| 2 | 39.8 | | 39.9 | 40.0 | 39.5 | 39.6 | | 40.5 | | 39.7 | | | 39.3 | 39.5 | 39.9 |
| 5 | 39.6 | | 39.6 | 39.8 | 39.6 | 39.3 | | 39.8 | 40.4 | 39.4 | | 37.2 | 38.7 | 39.3 | 39.5 |
| 10 | 39.6 | | 39.4 | 39.6 | 39.7 | 39.4 | | 39.7 | 38.9 | 39.6 | | 37.1* | 38.1 | 38.8 | 39.6 |
| 20 | | | | | | | | | 38.7† | | | | 36.7 | 38.1 | |
| | 0.8 mg. phosphorus (theoretical reading, 10.0 mm.). | | | | | | | | | | | | | | |
| 2 | 10.2 | 10.2 | 11.2 | 10.3 | 10.3 | | 10.4 | 10.7 | 14.2 | | 11.1 | 12.4 | 10.2 | 10.2 | 10.2 |
| 3 | 10.1 | 10.1 | 10.6 | 10.1 | 10.2 | | 10.0 | 10.2 | 11.9 | | 10.4 | 10.3 | 10.1 | 10.1 | 10.2 |
| 4 | 10.1 | 10.1 | 10.3 | 10.1 | 10.1 | | | 10.1 | 10.6 | | 10.3 | 10.1 | | | 10.1 |
| 5 | 10.1 | 10.1 | 10.2 | 10.1 | 10.1 | | 10.0* | 10.1 | 10.2 | | 10.2 | 9.9 | 10.0 | 10.0 | 10.1 |
| 10 | | | 10.1 | | | | | | 10.1 | | 10.0* | 9.8* | 9.9 | 10.0 | |
| 20 | | | | | | | | | | | | | 9.7 | 9.9 | |

1.6 mg. phosphorus (theoretical reading, 5.0 mm.).

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 5.4 | 6.3 | 7.3 | 6.1 | 6.1 | | 5.5 | 6.5 | 10.8 | | 6.9 | | 5.3 | 5.2 | 5.8 |
| 3 | 5.2 | 5.8 | 6.5 | 5.6 | 5.6 | | 5.3 | 5.6 | 8.2 | | 5.7 | 15.6 | 5.2 | 5.1 | 5.5 |
| 4 | 5.1 | 5.5 | 6.1 | 5.3 | 5.3 | | | 5.3 | 7.2 | | 5.3 | 10.0 | 5.1 | 5.1 | 5.3 |
| 5 | 5.1 | 5.4 | 5.9 | 5.2 | 5.2 | | 5.2 | 5.2 | 6.4 | | 5.2 | 5.9 | 5.1 | 5.1 | 5.2 |
| 6 | | 5.3 | 5.7 | 5.1 | | | | | 5.8 | | 5.1 | 5.3 | | | 5.1 |
| 8 | | 5.1 | 5.5 | | 5.1 | | 5.1 | 5.1 | 5.3 | | | 5.1 | | | |
| 10 | | | 5.4 | | | | | | 5.1 | | | | | | |
| 20 | | | 5.1 | | | | | | | | | | 5.0 | 5.0 | |

* The solution has a slight color when no phosphate is present.

† The solution has a purple tint, and does not exactly match the standard.

‡ Roughly corresponding to the composition of the filtrate from blood containing 3 mg. potassium oxalate per cc.[15]



[15] If oxalate did not penetrate the corpuscles, its concentration in the plasma would be much higher than in whole blood. In point of fact, the oxalate is about equally distributed between corpuscles and plasma. *E.g.*, 20 cc. of blood were mixed with 1 cc. of a solution containing 60 mg. of potassium oxalate (making the concentration in the mixture 2.86 mg. per cc.) and centrifuged. A sample of the plasma was precipitated with trichloroacetic acid as in the phosphate method, and the filtrate analyzed for oxalate following the general principles in common use for calcium estimations. 5 cc. of the filtrate (equivalent to 1 cc. of plasma) were found to contain 2.74 mg. of potassium oxalate.

working with material of uncertain nature, readings be taken after 5 and again after 10 minutes. If the two agree (assuming that the two solutions are practically alike in shade) there can be, as far as our experience goes, no doubt about the accuracy of the result. If, on the other hand, the reading has altered within that period, a third should be taken 5 minutes later. In case of any change occurring after 10 minutes, it is advisable to repeat the analysis with a smaller sample.[16]

That the numerous readings given in Table II might all represent the conditions finally adopted for the method here described, we were obliged, in order to avoid a delay of several months, to make them in warm weather (between 25 and 27°C.). The temperature coefficient of the color reaction as a whole happens to be distinctly high; a rise of 5° somewhat more than doubles the velocity. Except under extreme conditions, however, an allowance of 5 minutes is sufficient even at 20° if the reading is not less than 10 mm. At 20°, in the absence of interfering matter, 1.6 mg. of phosphorus (in a volume of 100 cc.) will take about 8 minutes to reach the proper reading. Agreement between consecutive readings, taken a few minutes apart, is perfectly reliable as a guide whatever the temperature may be.[17]

The superiority of aminonaphtholsulfonic acid is even more

[16] We wish to warn specifically against attempting to make the conditions in standard and unknown more uniform by altering the former (*e.g.*, by adding trichloroacetic acid and oxalate in the analysis of blood filtrates). Nothing can be gained by this procedure unless the two solutions are nearly equal in their phosphate content, and very little then. The danger lies in the chance of forfeiting what might otherwise be learned by taking two or more successive readings on the same solution; for, if the intensity of the standard is increasing too, any change in the unknown will be that much less readily detected. The advantage, also, of a single standard which serves for every purpose is far from being insignificant.

[17] Much time may be saved in special cases by the judicious use of warm water. For example, the trichloroacetic acid filtrate from a sample of nephritic plasma, when analyzed for inorganic phosphate in the customary manner (described below), gave a reading of 6.2. To a second sample of the same filtrate was added enough potassium oxalate to correspond to 3 mg. per cc. of plasma (in excess of that already present), followed by the usual reagents. Under these conditions, it would ordinarily take about half an hour to reach a constant reading. The unknown was therefore warmed for 5 minutes in a beaker of water kept at 35°. The reading was then 6.2, and underwent no further change.

pronounced in the case of solutions containing more than a trace of silicate. Partly because of the higher acidity[18] which we use—higher than is safe with hydroquinone—there will be no error in the presence of 25 mg. of silicon to 1 mg. of phosphorus if the color is read within 5 minutes. When larger amounts of silica are present, accurate analyses can still be made (even with 200 times as much silicon as phosphorus) by adding to the unknown solution (before the molybdate) 1 cc. of 10 N sulfuric acid in case the solution is to be diluted to 100 cc., or a proportionate amount for smaller volumes. The total sulfuric acid concentration will then be 0.6 N, and the synthesis and subsequent reduction of silicomolybdic acid about one-eighth as fast as in 0.5 N acid.

Nitrite, in amounts too small to produce a noticeable error, will confer a yellowish tinge[19] on the solution, easily perceived when examined in the colorimeter.[20] The permissible quantity of nitrite can be made considerably greater by using more bisulfite; if no yellow color can then be seen when making the reading, the result will be correct. In dealing with solutions containing much nitrite, the formation of gas bubbles offers another warning signal—in the colorimeter they make the solution appear gray by comparison with the standard. If the colorimeter cup is removed and gently rotated, the gas can all be liberated and accurate readings made, provided that enough bisulfite has been added.[21]

Sodium chloride and potassium nitrate both slightly retard the reduction. We have not attempted to establish definitely the upper limits that are safe in these two cases—it is sufficient that a good deal may be present without perceptible effect. For ashing purposes, as much as 5 gm. of a mixture of sodium carbon-

[18] In our method the sulfuric acid concentration in the final mixture is 0.5 N; in Briggs' urine method about 0.27 N. At the latter acidity, aminonaphtholsulfonic acid will not give proportionate readings, while in 0.5 N acid hydroquinone will do so in the course of time (a matter of some hours). When the acidity is too low, either molybdic acid itself is reduced, or traces of silicate in the reagents interfere—we are not sure which is the more important factor.

[19] A yellow color with various other naphthol derivatives has been used as a qualitative test for nitrite (*cf.* Vági, S., *Z. anal. Chem.*, 1925, lxvi, 14).

[20] With hydroquinone there is no such guide; the color intensity is affected by less nitrite than is required to modify the tint.

[21] For each cc. of M nitrite present, the solution should contain about 3 cc. of 15 per cent bisulfite.

ate and potassium nitrate may be used (for quantities of phosphorus ranging from 0.2 to 1.6 mg.), and that is more than enough to cover ordinary needs.[22]

Ammonium sulfate has a more pronounced effect than either of the salts just mentioned. Its presence, however, can be recognized by the purple shade of the solution, which appears when the concentration of ammonium sulfate is still too low to change the colorimeter reading.

The effect of ferric chloride is quite different from that of anything else that we have tested. In solutions which contain tervalent iron, the development of color could almost be called a "clock reaction." This feature is especially prominent in phosphate solutions much stronger than the standard, containing fairly large amounts of iron. There is then at first only a pale green color, which very gradually becomes deeper, though still green. After a latent period, which is longer the more iron there is present, the color suddenly becomes much more intense, and at the same time changes from green to blue. The time required for the reaction to reach any arbitrarily selected stage (*e.g.* the point where a printed letter observed through a given thickness of the solution becomes invisible) can be reproduced under uniform conditions within a few seconds. Once this final stage of the reduction has begun, the color reaction then proceeds with practically the same velocity that it would have shown if no iron had been added, as may be clearly seen from the experiment (Table II) with 1.6 mg. of phosphorus and 15 mg. of iron.

A cursory examination of Table II will make it evident that, in the presence of any substance which retards the color reaction, the time consumed in reaching the correct reading is longer the higher the phosphate concentration.[23] Although we have included

[22] The nitrate, on ignition, is reduced to nitrite. It is assumed that most of the nitrous acid will be disposed of by evaporating with hydrochloric acid.

[23] This point might well be borne in mind in connection with the influence of foreign material on any colorimetric method. The mere fact that the color intensity of the standard is no different in the presence of a given substance has no bearing on what will happen if the solution is stronger or weaker than the standard. The truth of this is demonstrated in nearly every column of Table II.

solutions up to four times the strength of the standard in our tests, the reading of colors so far apart as that is not to be recommended as a routine practice, at least without special care in adjusting the zero points of the colorimeter. The proportionality range is nevertheless extremely wide, as color reactions go, and the knowledge that it does extend so far has in our hands prevented many unnecessary repetitions. By the use of small corrections, the accuracy of the reading can be made to compare favorably with that attainable in most colorimetric methods over a much narrower range.

We are not sure that the kind of colorimeter is immaterial. All the readings reported in this paper were obtained with the Bausch and Lomb "biological type," and with our instrument the correction is about 1 per cent when the reading is 10 mm., the same when it is 40 mm., and 2 per cent or slightly less for readings as low as 5 mm. That is, the averages of many consistent readings on solutions of the three strengths indicated are (to the nearest tenth) 10.1, 39.6, and 5.1 mm., respectively.

Having discussed what we have learned of the limitations of the method in the analysis of known mixtures, we may now proceed to its application to biological material, particularly to urine and to blood.

### *Solutions Needed.*

*10 N Sulfuric Acid.*—450 cc. of concentrated sulfuric acid added to 1300 cc. of water.

*Molybdate I.*—2.5 per cent ammonium molybdate in 5 N sulfuric acid. Dissolve 25 gm. of the salt in 200 cc. of water. Rinse into a liter volumetric flask containing 500 cc. of 10 N sulfuric acid. Dilute to the mark with water and mix.

*Molybdate II.*—2.5 per cent ammonium molybdate in 3 N sulfuric acid. Prepared as above, but with only 300 cc. of 10 N sulfuric acid. (To be used only with blood filtrates in the determination of inorganic phosphate.)

*Molybdate III.*—2.5 per cent ammonium molybdate in water. As soon as any considerable amount of sediment (ammonium trimolybdate) has appeared in this solution, it should be discarded.

*10 Per Cent Trichloroacetic Acid.*—The quality of this reagent is of great importance. One brand that we have tried contains some unknown impurity which retards the color development to a most pronounced degree. Merck's U.S.P. product, which we now use, is free from any such contamina-

tion, but contains a trace of phosphate. The amount of this must be determined in each sample,[24] or else the reagent purified by distillation.[6]

*Standard Phosphate (5 Cc. = 0.4 Mg. P).*—Dissolve 0.3509 gm. of pure monopotassium phosphate in water. Transfer quantitatively to a liter volumetric flask, add 10 cc. of 10 N sulfuric acid, dilute to the mark, and mix. The standard keeps indefinitely.

*15 Per Cent Sodium Bisulfite.*—The solution must be free from turbidity before it can be used. Freshly prepared sodium bisulfite solutions may not filter clear, in which case 2 or 3 days standing (before filtering) will be necessary. Keep well stoppered.

*20 Per Cent Sodium Sulfite.*—Because it is likely to be of better quality, we prefer to use the crystalline sulfite ($Na_2SO_3 \cdot 7H_2O$). Dissolve 200 gm. of this in 380 cc. of water. Remove any suspended matter by filtration, and keep stoppered.

*0.25 Per Cent Aminonaphtholsulfonic Acid.*—Dissolve 0.5 gm. of the dry powder (see next section) in 195 cc. of 15 per cent sodium bisulfite, add 5 cc. of 20 per cent sodium sulfite, stopper, and shake until dissolved. If the bisulfite solution is old, more than 5 cc. of sulfite will be needed—in that event add more sulfite 1 cc. at a time, shaking after each addition, until solution is complete. This reagent can be prepared in a few minutes (the powder need not be very accurately weighed), and if not left exposed to the air it should keep about 2 weeks. The solution is more stable the higher its acidity, hence no more sulfite should be added than is needed to dissolve the reducing agent.

*1, 2, 4–Aminonaphtholsulfonic Acid.*

This may be prepared from β-naphthol according to Folin's directions, with a single alteration. The final product, after washing with cold water,[25] still contains some colored material. To remove this, the crystals

[24] Arrange three tall beakers of 150 cc. capacity on a piece of white paper. Into one of these (A) put 100 cc. of water. In a second beaker (B) mix 85 cc. of water, 10 cc. of Molybdate I, and 4 cc. of 0.25 per cent aminonaphtholsulfonic acid; the result should be a solution practically as colorless as water, without a trace of blue (otherwise one or more of the reagents already added contains phosphate). To the third beaker (C) add 40 cc. of the trichloroacetic acid solution, 45 cc. of water, 10 cc. of Molybdate II, and 4 cc. of the sulfonic acid reagent, stirring thoroughly with a clean glass rod. Into B now run 1 cc. of a dilute phosphate solution containing 0.005 mg. of phosphorus per cc., and mix well. Proceed in the same way, adding 1 cc. of this phosphate solution at intervals of not less than 2 minutes, until B and C appear to have the same color when examined from above. The volume of phosphate solution which must be added to bring this about, multiplied by 0.05, is the correction (in mg. per 100 cc.) to be subtracted from the result in the analysis of blood.

[25] Folin, O., foot-note 13, p. 388, step 8.

on the filter, while still wet, should be further washed with alcohol as long as any color is extracted.

The reagent may also be obtained in satisfactory condition by one recrystallization of "technical" aminonaphtholsulfonic acid (Eastman Kodak Co.), as follows: Heat 1000 cc. of water to about 90°, and dissolve in it 150 gm. of sodium bisulfite and 10 gm. of crystalline sodium sulfite. To this mixture add 15 gm. of the crude sulfonic acid, and shake until all but the amorphous impurity has dissolved. Filter the hot solution through a large paper (about 32 cm.), cool the filtrate thoroughly under the tap, and add to it 10 cc. of concentrated hydrochloric acid. Filter with suction, wash with about 300 cc. of water, and finally with alcohol until the washings are colorless.

The purified sulfonic acid should be dried in air with the least possible exposure to light, then powdered and transferred to a brown bottle.

*Determination of Inorganic Phosphate in Urine.*

*Description of Method.*—Measure into a 100 cc. volumetric flask enough urine to contain between 0.2 and 0.8 mg. of inorganic phosphorus (usually 1 or 2 cc.).[26] Add water to bring the total volume to 70 cc., followed by 10 cc. of 2.5 per cent ammonium molybdate made up in 5 N sulfuric acid (Molybdate I), and 4 cc. of 0.25 per cent aminonaphtholsulfonic acid. After the addition of each reagent, the solution should be mixed by gentle shaking. At the same time transfer to a similar flask 5 cc. of the standard phosphate solution (containing 0.4 mg. of phosphorus), 65 cc. of water, and the same reagents that were added to the urine sample. Dilute the contents of each flask to the mark, mix, and compare in the colorimeter after 5 minutes. With the standard set at 20 mm., 8 divided by the reading will give the inorganic phosphorus content of the sample in mg.

We have so far not encountered any urine in which the rate of reduction by the sulfonic acid is seriously retarded, but in case the reading falls below 10 mm., it should be controlled by a second reading taken a few minutes later. Unsatisfactory results with hydroquinone lead us to suspect that urine may sometimes contain interfering substances.

*Results.*—The above procedure has been checked against the titration method described some time ago by one of us[27] (based on

[26] As suggested earlier, accurate results can be obtained, with proper care, using as much as 1.6 mg. of phosphorus.

[27] Fiske, C. H., *J. Biol. Chem.*, 1921, xlvi, 285.

the alkalimetric titration of $MgNH_4PO_4$). The results (Table III) are all that could be hoped for.[28]

*Albuminous Urine.*—According to Briggs, if turbidity appears on adding the molybdate reagent to the (diluted) urine sample,

TABLE III.

*Analysis of Human Urine.*

| Urine No. | Volume per hr. | Titration method. | | | | Colorimetric method. | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Urine. | 0.1 N HCl neutralized. | Inorganic P. | Inorganic P per hr. | Urine. | Reading. | Inorganic P. | Inorganic P per hr. |
| | *cc.* | *cc.* | *cc.* | *mg.* | *mg.* | *cc.* | *mm.* | *mg.* | *mg.* |
| 1 | 109 | 10 | 2.49 | 3.86 | 42.1 | 1.0 | 20.7 | 0.386 | 42.1 |
| | | | | | | 2.0 | 10.3 | 0.778 | 42.4 |
| 2 | 29 | 5 | 1.53 | 2.37 | 13.7 | 0.6 | 28.5 | 0.281 | 13.6 |
| | | | | | | 1.0 | 17.1 | 0.468 | 13.5 |
| 3 | 24 | 5 | 2.67 | 4.14 | 19.9 | 0.4 | 24.2 | 0.331 | 19.9 |
| | | | | | | 1.0 | 9.7 | 0.825 | 19.8 |
| 4 | 49 | 7 | 2.81 | 4.36 | 30.5 | 0.6 | 21.6 | 0.370 | 30.2 |
| | | | | | | 1.0 | 13.0 | 0.616 | 30.2 |
| 5 | 40 | 7 | 2.66 | 4.13 | 23.6 | 0.6 | 22.7 | 0.352 | 23.5 |
| | | | | | | 1.0 | 13.6 | 0.588 | 23.5 |
| 6 | 39 | 5 | 2.37 | 3.68 | 28.7 | 0.5 | 21.7 | 0.369 | 28.8 |
| | | | | | | 1.0 | 10.9 | 0.734 | 28.6 |
| 7 | 18 | 5 | 2.40 | 3.72 | 13.4 | 0.5 | 21.8 | 0.367 | 13.2 |
| | | | | | | 1.0 | 10.9 | 0.734 | 13.2 |

the precipitate is to be removed by centrifugation while the color is developing. The formation of a precipitate at this point must generally mean that protein is present—at least we know of no

[28] We have never been able to duplicate them by the Briggs procedure. Briggs compared his method with Bell and Doisy's on three samples of urine, and was evidently satisfied with discrepancies as large as 7 per cent. Errors of 3 per cent, in our experience, are common when hydroquinone is used, and sometimes the result may be much farther off.

other common constituent of urine that is likely to produce it[29]—and in that event removing the suspended matter by centrifugation is most emphatically not the thing to do. No matter how slight the turbidity may be, if it can be thrown down at all by centrifuging, it will be seen to be intensely blue if our reducing agent has been used. Very roughly, 1 mg. of protein will precipitate 0.01 mg. of phosphorous (as protein phosphomolybdate), and diminish the color of the supernatant fluid by that amount. No very large quantity of protein would be necessary to carry down every trace of phosphate, leaving the solution quite devoid of color.

As a matter of fact, our molybdic acid reagent is not a very delicate protein precipitant (far less so than phosphomolybdic acid), and with the majority of albuminous urines it would give no protein precipitate at all if phosphate were not present.

If the molybdate reagent causes any sign of cloudiness, the determination must be begun again with a new sample from which all protein has been removed. For this purpose, to an accurately measured amount of urine add four times its volume of 10 per cent trichloroacetic acid, stopper, shake, and filter through an ashless paper. 2 to 10 cc. of the filtrate (equivalent to 0.4 to 2 cc. of urine) should ordinarily be the right amount to take. In case the urine happens to be so dilute that more than 10 cc. of filtrate must be used, the analysis should be made by the slightly modified method outlined below for blood filtrates.

*Determination of Inorganic Phosphate in Blood.*

*Anticoagulant and Protein Precipitation.*—The inhibiting effect of such amounts of oxalate as might be used to prevent coagulation[30] was thought by Briggs to be avoided when the blue color is read in acid solution. The real reason, however, why his method is less sensitive than Bell and Doisy's to oxalate, etc., is that 30 minutes (instead of 5) are allowed for the development of color. The subsequent addition of carbonate—as in the Bell and Doisy

[29] Normal urine, if not previously diluted, may give a precipitate with the molybdate reagent. Turbidity not of protein nature is hence a possibility in the case of material very poor in phosphate (*e.g.* the urine of many Herbivora, or in special circumstances that of other animals), but we have not yet had occasion to observe it.

[30] Denis, W., and von Meysenbug, L., *J. Biol. Chem.*, 1922, lii. 1.

method—cannot make any difference, for the reduction of phosphomolybdic acid takes place in acid solution anyway.

Whether the necessary amount of oxalate, by itself, does or does not influence the color intensity is really of no particular moment. Blood filtrates, as prepared for this determination, always contain both oxalate (or some other anticoagulant) and trichloroacetic acid, and they affect the color reaction additively—a point of some importance which seems to have been wholly overlooked. Martland and Robison[1] find, in fact, that trichloroacetic acid alone, in the concentration which Briggs' directions call for, is not without effect, and recommend the use of less; *i.e.*, enough to make the final concentration in the filtrate 2.5, instead of 4, per cent. However necessary this modification may be to give correct results, by Briggs' method, with phosphate solutions to which trichloroacetic acid has been added, it is unfortunately not safe to use with blood. Even 4 per cent trichloroacetic acid will sometimes fail to remove the protein completely, for we have often encountered turbidity on adding molybdic acid to such filtrates. The turbidity may be too slight to strike the eye in as small a volume of filtrate as 5 cc., except on careful examination against a dark background. But if a larger volume of the filtrate is treated in the usual way, definite turbidity is much too common an occurrence. Even if it cannot be clearly seen in 5 cc. of filtrate, it may still make the result too high by at least 0.2 mg. of phosphorus per 100 cc. of blood or plasma; at the same time it may so modify the shade that accurate matching is impossible. Diminishing the concentration of trichloroacetic acid to 2.5 per cent naturally does not improve the situation.

To be sure of quite protein-free filtrates, we have found it necessary to increase the trichloroacetic acid materially, so that the filtrate contains 8 per cent. Other advantages are then gained as well, for the filtration is several times as rapid, and the yield of filtrate greater.[31] The effect of this additional acid alone on the rate of color development in our method would not be very serious, but since oxalate is present too the time may be undesirably prolonged. Here again we must insist that adding oxalate and trichloroacetic acid to the standard is not the way to get around the difficulty. Instead, we add to the blood (or plasma) filtrate—

[31] *Cf.* Hiller, A., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, liii, 253.

but *not* to the standard—a special molybdate reagent (Molybdate II) made up with less sulfuric acid. The acidity is then about the same in standard and unknown, and the color reaction proceeds quite rapidly enough. The combined effect of trichloroacetic acid and oxalate under these conditions is shown in the last column of Table II. Citrate has substantially the same effect as oxalate.

In view of the rise in inorganic phosphate[32] that begins as soon as blood is laked,[33, 34] the usual method of adding the protein precipitant to blood previously hemolyzed with water has had to be abandoned. Lawaczeck[33] avoids this trouble by laking with hydrochloric acid (in conjunction with the Schenck method of precipitation); and Martland and Robison[6] have ingeniously adapted the same principle by laking with dilute (1 per cent) trichloroacetic acid, which stops the enzyme action without precipitating protein. From the standpoint of eliminating the source of error mentioned, this is quite ideal, but it is cumbersome. Having had occasion, in the course of experiments on animals, to draw and precipitate blood with the greatest possible rapidity—in order to be free to go on with other operations—we were glad to find that laking is not necessary at all. Whole blood may be run directly into a solution of trichloroacetic acid with no danger of incomplete extraction.[35] The manipulation is then even simpler than in the old (and unreliable) method of laking first with water, for there is only one liquid (besides the blood) to be measured out, and that may be done before the blood is drawn. A number of comparisons with Martland and Robison's method of precipitation[36] have been made, without finding any difference. We shall

[32] Caused by the enzymotic hydrolysis of phosphoric acid esters.

[33] Lawaczeck, H., *Biochem. Z.*, 1924, cxlv, 351.

[34] Martland, M., Hansman, F. S., and Robison, R., *Biochem. J.*, 1924, xviii, 1152.

[35] This applies even to the most abundant crystalloid constituent of corpuscles (*i.e.* potassium), for the total base of corpuscle suspensions can be quantitatively extracted with trichloroacetic acid without laking (Fiske, C. H., unpublished experiments). It probably holds, therefore, for most substances in that category, and perhaps for all. To what extent the same is true with other protein precipitants we are not prepared to say. The high osmotic pressure of the trichloroacetic acid solution used is no doubt a factor in causing the corpuscles to expel their contents.

[36] Modified to make the final concentration of trichloroacetic acid 8 per cent.

give but one example—a specimen of blood which happened to be used for the investigation of some other points besides (Table IV).

One of these points is the question of the recovery of added phosphate. For this as the sole criterion of reliability for any method of blood analysis—as some consider it to be—we have not much respect.[37] It does, however, in this case have some bearing on the accuracy of the determination when the level is abnormally high (as in nephritic blood). 20 mg. of added phosphorus (in the

TABLE IV.

*Analysis of Dog Blood.**

| Laked with 1 per cent trichloroacetic acid.† | | Direct precipitation without laking. | | Remarks. |
|---|---|---|---|---|
| Inorganic P. | Total acid-soluble P. | Inorganic P. | Total acid-soluble P. | |
| 2.85 | | 2.81 | | |
| | | 23.4 | | 20 mg. P added.‡ |
| | 16.8 | | 16.9 | 5 cc. filtrate ashed with 5 cc. 5 N $H_2SO_4$ and 1 drop $HNO_3$. |
| | | | 16.9 | Same, but overheated.§ |
| | | | 16.8 | 1 cc. filtrate ashed with 1 cc. 5 N $H_2SO_4$ and 1 drop $HNO_3$. |
| | | | 16.7 | Dry ashing. |

* The blood was precipitated at a dilution of 1:5, the final concentration of trichloroacetic acid being 8 per cent. The figures in the table represent mg. of phosphorus per 100 cc. of blood.

† Martland and Robison's method.

‡ 10 cc. of blood were mixed in a small Erlenmeyer flask with 1 cc. of a $KH_2PO_4$ solution containing 2 mg. of phosphorus. As much of the mixture as possible was run into 39 cc. of 10 per cent trichloroacetic acid, and the residue in the small flask then thoroughly mixed with the precipitated blood.

§ As described in text.

form of $KH_2PO_4$) are quantitatively recovered by our precipitation method, even at a 1:5 dilution. The particular example cited in support of this (Table IV) is of interest in another way. 20 mg. of phosphorus were added; the recovery was 23.4 − 2.8 =

[37] A more reliable method of determining whether extraction is complete is to compare filtrates made from ordinary blood (*i.e* with nothing added) at different dilutions, say 1 : 5 and 1 : 10. This we have done on numerous occasions; the result at the higher dilution is never any more.

20.6 mg., or 3 per cent too much. The excess, within the limit of error, is what should be expected when account is taken of the volume occupied by the protein precipitate. When blood containing 20 per cent of protein is precipitated at a dilution of 1:5, the concentration of the filtrate should be about 4 per cent too high. For ordinary comparative analyses this error may be neglected, but in some cases it must be taken into consideration and corrected for, or a higher dilution used.[38]

In Table IV will be found also a few figures for total acid-soluble phosphorus. The result is in this case likewise no different when the laking is omitted.

*Method for Inorganic Phosphate.*—Transfer to an Erlenmeyer flask 4 volumes of 10 per cent trichloroacetic acid. While the flask is being gently rotated, run in 1 volume of blood,[39] plasma, or serum—as the case may be—from a pipette calibrated for delivery (not contents). Close the mouth of the flask with a clean, dry rubber stopper, and shake vigorously a few times. Filter through an ashless paper.[40]

Measure 5 cc. of the filtrate into a tube graduated at 10 cc. or a 10 cc. volumetric flask. Add 1 cc. of 2.5 per cent ammonium molybdate in 3 N sulfuric acid (Molybdate II), and finally (after mixing) 0.4 cc.[41] of the usual sulfonic acid reagent. Dilute to the mark and mix. The standard, to be prepared as nearly as possible at the same time, is identical with the standard used for urine (0.4 mg. of phosphorus in a volume of 100 cc., or 0.2 mg. in a 50 cc. flask with half as much of each reagent), so blood and urine may be read against the same solution.[42] It should be noted that the molybdate reagent added to the standard is always the one containing 5 N sulfuric acid (Molybdate I), and is different from that used for the blood filtrate. The purpose of this, as stated

[38] *E.g.*, in calculating the composition of the corpuscles from analyses of whole blood and of plasma (which contains less protein).

[39] Oxalate is the most suitable anticoagulant. Use 2 (or at the most 3) mg. of potassium oxalate per cc. of blood.

[40] The mixture may be filtered at once.

[41] 0.4 of the number of drops contained in 1 cc. is close enough.

[42] With only 10 or 15 cc. of standard solution, as Briggs for example recommends, we are unable to see how the standard can first be matched against itself, as it must be always to get dependable results.

elsewhere, is to compensate for the high concentration of trichloroacetic acid in the filtrate.

The reading, as with urine, may be made in about 5 minutes, but it should be repeated a few minutes later if the color is particularly strong. To calculate the result in mg. of phosphorus per 100 cc. of blood or other fluid (the standard being set at 20 mm.), divide 80 by the reading. From the figure so obtained subtract the correction for any phosphate which the trichloroacetic acid may contain.

An inorganic phosphorus content of 2 mg. per cent is about the lower limit for convenient reading against the standard recommended, and a weaker color—such as would be obtained by using half as strong a standard—cannot be read so accurately. Hence we suggest, as perhaps the least objectionable arrangement when the phosphate content of the blood is very low, the addition of a known amount of phosphate to the filtrate. This may be done, when a low result can be anticipated, before introducing the reagents. Otherwise, if the reagents are already mixed with the blood filtrate and the color is seen to be unusually weak, phosphate may be added then, before diluting to the mark—the less delay of course the better, but any time within 5 minutes will do, provided that twice as long a period is allowed before the final reading. A suitable amount of phosphorus to add is 0.016 mg., or 1 cc. of a solution made by diluting 20 cc. of the regular standard to 100 cc. The calculation may then be made in the customary manner, and 1.6 mg. per cent subtracted from the result.

By way of illustration, the following check on this procedure may be mentioned. A solution of phosphate, oxalate, and trichloroacetic acid was prepared to simulate the filtrate from blood containing 0.40 mg. of inorganic phosphorus per 100 cc. The usual reagents were added to a 5 cc. sample of this solution and to the standard. 5 minutes later 0.016 mg. of phosphorus was added to the unknown. After 9 minutes, the corrected colorimeter reading was 38.0 mm. The calculation follows.

$80 \div 38.0 = 2.11$ mg. of phosphorus per 100 cc. From this must be subtracted a correction of 0.12 mg. for the phosphate in the trichloroacetic acid, as well as one of 1.6 mg. for the phosphate added in the course of the analysis. The final result becomes $2.11 - 0.12 - 1.60 = 0.39$ mg. per cent, instead of the theoretical figure 0.40.

Subsequent readings, made at intervals for the next half hour, showed no further change.

*Determination of Total Phosphorus.*

*Destruction of Organic Matter.*—Much has been written of the danger of loss of phosphate, in one way or another, in the course of the wet ashing process. The danger is without the slightest doubt a real one, at least in the case of material difficult to digest. But when no such difficulty presents itself (*e.g.* in ashing protein-free blood filtrates) we are convinced that the loss is brought about by trying to digest with so little sulfuric acid that the tube is nearly dry; and probably then by quite unnecessary overheating.

In making total phosphorus determinations by the colorimetric method in this laboratory, advantage has long been taken of the device which Martland and Robison[6] now say is necessary for accurate results. That is, the entire amount of sulfuric acid which is later to be used in developing the blue color is added before the digestion is begun, and an ammonium molybdate solution which contains no sulfuric acid is substituted for the usual acidified reagent. If as much as 4 or 5 cc. of protein-free filtrate can be spared for the determination of total acid-soluble phosphorus in whole blood, the equivalent of about 0.7 cc. of concentrated sulfuric acid (5 cc. of 5 N) can then be used for ashing, with not the slightest danger of losing phosphate in the process. In the second total phosphorus determination recorded in Column 4 of Table IV, the mixture was heated much longer than necessary. After the solution had become entirely colorless, it was boiled for an additional period of 10 minutes, with the micro burner flame (1 cm. in length) just touching the bottom of the test-tube. The tube, throughout this time, was filled with sulfuric acid fumes to within 5 cm. of the top, and yet there was no loss. Substantially the same result was found with a 1 cc. sample of the same filtrate, which was ashed with only one-fifth as much sulfuric acid in a small test-tube, as described below. As a further check, one more analysis was made, this time by dry ashing with a fusion mixture. All four determinations gave the same result within the limit of error of the reading.

*Method for Total Acid-Soluble Phosphorus in Blood.*—5 cc. of the trichloroacetic acid filtrate should be used if possible. Boil this down, over a micro burner, in a large lipped Pyrex test-tube (200 by 25 mm.) with 5 cc. of 5 N sulfuric acid (or 2.5 cc. of 10 N) and a piece of quartz to prevent bumping. The bottom of the

tube should be about 2 cm. above the burner tip. As soon as charring can be seen, or fumes appear, turn the flame down very low, so that the mixture barely boils, and heat until there is no further blackening. Now add 1 drop of nitric acid so that it runs down the wall of the test-tube—it should not fall directly into the digestion mixture. If the color does not promptly disappear, add another drop of nitric acid in the same manner, and continue in this way until there is no color left. Nothing is gained by using a large amount of nitric acid. Ordinarily a single drop will be enough, and then about 30 seconds further boiling with the same low flame (to remove most of the nitric and nitrous acids left) will complete the ashing process.

Cool the tube under the tap, rinse the contents into a 50 cc. volumetric flask with 35 cc. of water, add 5 cc. of Molybdate III (2.5 per cent ammonium molybdate in water alone) and 2 cc. of the reducing agent. Dilute to the mark, and proceed as usual, reading against the standard that has been described before. 400 divided by the reading will give the desired result in mg. per 100 cc. of blood.

The analysis may be made with 1 cc. of filtrate, using 1 cc. of 5 N sulfuric acid. The procedure is then otherwise the same as that described above, except that the final dilution must be 10 cc. (instead of 50) and the reagents diminished in proportion. It is safer, in this case, to use a smaller test-tube (about 10 mm. in diameter) for the digestion.[43] The most probable cause of loss of phosphate is superheating at the edge of the meniscus, which should consequently be as far removed as possible from the source of heat. Digesting in 50 cc. flasks, as recommended by Martland and Robison,[6] must certainly exaggerate the danger, and doubtless explains the trouble they experienced in trying to ash blood filtrates with a few drops of concentrated sulfuric acid.

*Other Applications.*

The colorimetric method having now been placed upon a sounder basis, it may be applied with safety to the analysis of any sort of

[43] To prevent excessive foaming, some stable and not too volatile inhibiting agent (*e.g.* phenyl ether) may be found helpful. It should be added from a tube drawn out to a fine capillary, so that the drops are very small. Only 1 drop should be used to start with, and no more added later unless the first drop has all been driven off before the foaming stage is passed.

biological material if the precautions stated are attended to. To give, in full detail, directions for meeting every possible contingency would take much space and is unnecessary.

In the determination of total phosphorus, for example, the main question in any case is how to ash most easily and safely—merely a matter of selecting one of the various wet and dry incineration methods that have long been used in gravimetric work. Urine, blood, lipoid extracts, etc., may be treated according to the directions given for blood filtrates, except that more nitric acid will naturally be needed. Occasional checks by some dry ashing method will be found useful—in no other way can one's confidence in the wet method be maintained and its limitations clearly recognized.

Dry incineration as a routine practice also has its place. When large quantities of organic matter must be dealt with, it is not only more reliable but quicker. If there is a relatively large volume of water to be removed from the sample being analyzed, dry ashing is sometimes simpler anyway, for evaporation in an open dish may be less trouble than boiling off the water in a test-tube. With blood or plasma filtrates, the trichloroacetic acid should first be driven off on the water bath; the small amount of organic matter left can be destroyed by ignition with a few cgm. of sodium carbonate.

Nitrate must be added to more refractory material, and here no rigid rules can be laid down. In case the residue is more or less adhesive, as with urine, magnesium nitrate alone (1 cc. of 10 per cent) is very satisfactory. For tissues, or for comparatively pure products, it is not so good as the carbonate-nitrate fusion mixture of Berzelius, which was apparently first used for the quantitative analysis of biological products by Falck[44] in 1873.

The phosphate method we propose is so little affected by silicate that porcelain dishes may be used for the ignition. Our experience with them is so far limited to fusion mixtures of low melting point. For this reason, and because porcelain is not all alike, a blank should be run on every dish before using it for phosphorus determinations. The porcelain will be attacked to some extent, and silicate dissolved, but the subsequent evaporation with hydrochloric acid (to neutralize the carbonate) will

[44] Falck, F. A., *Ber. chem. Ges.*, 1875, viii, 12.

change most of it to insoluble silica, which is easily removed; the small amount remaining in solution we have never found to be enough to interfere. If any doubt exists upon this point, successive readings will decide it too, for silica enough to make the analysis inaccurate will cause a progressive growth in color intensity lasting many hours (see Table II).

Obviously, when nitrate fusions are made in platinum, as much as possible of the ash must be dissolved in water and transferred to glass or porcelain before adding hydrochloric acid.[45] The acid should be used to remove whatever undissolved material (calcium and magnesium phosphates) may be left adhering to the platinum dish. The entire solution, inclusive of the acid washings, is then evaporated to dryness in a beaker or a porcelain dish (covered with a watch-glass as long as carbon dioxide is evolved), the residue transferred with water to a volumetric flask, and the color developed as in any other case. Silica may be removed by centrifugation, if necessary, before the color is read.

Finally, we may mention a special problem which was always troublesome to handle by the older phosphate methods. This is the determination of traces of phosphorus in the presence of a large excess of carbonaceous matter. Outstanding examples are the determination of organic phosphorus in urine and the analysis of proteins containing very small amounts of phosphorus —whether an integral part of them or present as impurity. It is in cases of this kind that advantage may profitably be taken of the fact that the blue color is intensified by heating,[46, 9] and here too aminonaphtholsulfonic acid is the best reagent known to us. We do not believe that the heating method can be made quite as accurate as the one with which this paper is concerned, and we use it only when the latter is excluded. The details will be published in due time.

[45] One apparent advantage of magnesium (or calcium) nitrate is the fact that ignition decomposes it to the oxide, not the nitrite. Evaporation with hydrochloric acid directly in the platinum dish will consequently cause no serious damage. We hesitate to recommend this as a regular thing, however. If the sample contains salts of sodium or potassium, some nitrite may be left undecomposed, and injury to the platinum in time may be appreciable.

[46] Benedict, S. R., and Theis, R. C., *J. Biol. Chem.*, 1924, lxi, 63.

# VITAMIN STUDIES.

## XI. INORGANIC BLOOD PHOSPHORUS AND BONE ASH IN RATS FED ON NORMAL, RACHITIC, AND IRRADIATED RACHITIC DIETS.*

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In the course of our studies on the effect of commercial methods of evaporation on vitamins A, B, and C in cow's milk, it appeared desirable to take advantage of our supply of experimental milks and extend our studies to include the antirachitic factor. Inasmuch as x-ray and histological facilities were not available, it became necessary to resort to chemical methods, as a means of ascertaining the physiological conditions of our rats, so far as calcium and phosphorus deposition is concerned.

In the preliminary feeding trials we used the Sherman-Pappenheimer ration (1) but this was finally discarded in favor of a "yellow corn ration" devised by Dr. Harry Steenbock of the Wisconsin Experiment Station. We feel that the latter ration has given more satisfactory results because of its superior protein complex and its higher content of vitamins A and B. This ration will be described at another point in this paper.

Examination of the literature showed that little work had been done on the chemical composition of blood and bones of rats at different ages and, inasmuch as the rachitic rations, used by other investigators, have varied in their chemical composition, particularly with reference to calcium and phosphorus, it was deemed advisable to make a preliminary study on our rats under the conditions existing in our laboratory with the view of determining

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the composition of blood and bone at various ages, using the new Steenbock rachitic ration.

It was decided that determinations of inorganic blood phosphorus should be made because previous investigations (2) have shown that a knowledge of the fluctuations of inorganic phosphorus in blood might be helpful in making our interpretations.

Work of McCollum (3), Steenbock (4), and others has indicated that the determination of the ash content of bones is even more helpful in arriving at conclusions regarding the utilization of calcium and phosphorus by the rat. For this reason, it was decided that this determination should be made, using the extraction method of Steenbock.

## EXPERIMENTAL.

While it has not been our general practice, it became necessary to resort to group feeding for the reason that it was difficult to obtain sufficient blood from a single rat, especially during the early stages of the experiment when the rats were small. Consequently, each cage contained not less than four rats, while some cages, which contained rats which were to be removed from the experiment during the first few weeks, contained a larger number, the total number of animals being 103. The rats were placed on experiment at an age of about 21 days, no animal weighing less than 35 or more than 40 gm.

Three types of rations were fed; Ration A being the normal ration which is used for our breeding stock; Ration C being Steenbock's yellow corn ration, and Ration B, Steenbock's ration which had been irradiated for 15 minutes with the Cooper-Hewitt "Uviarc" quartz mercury vapor lamp at a distance of 2 feet.

Ration A consisted of the following:

| | *per cent* |
|---|---|
| Wheat | 45.0 |
| Corn | 17.0 |
| Rolled oats | 4.9 |
| Linseed meal | 8.0 |
| Bran | 2.5 |
| Dried meat scraps | 5.0 |
| Powdered skim milk | 10.0 |
| Sodium chloride | 1.0 |
| Calcium carbonate | 1.5 |
| Ferric citrate | 0.1 |
| Alfalfa meal | 5.0 |

These ingredients were ground and fed with fresh whole milk (*ad libitum*) and fresh greens were fed twice each week.

Ration C, Steenbock's yellow corn ration, consisted of the following ingredients:

| | per cent |
|---|---|
| Yellow corn (ground) | 76.0 |
| Wheat gluten | 20.0 |
| Calcium carbonate | 3.0 |
| Sodium chloride | 1.0 |

As stated above, Ration B was the same as Ration C except that it was irradiated with ultra-violet light.

The experiment was conducted for 8 weeks and at least four animals were removed each week from each group represented by the rations described. When the rats were removed at the end of each week, they were anesthetized and the blood was obtained by cutting the jugular vein. The individual blood samples of each experimental group were added together and set aside in a refrigerator overnight for the determination of inorganic phosphorus. The femurs were removed, scraped free from adhering tissue, and composited into single experimental samples, air-dried, and extracted with alcohol and ether. After extraction, the composite weekly samples of bones were dried in an oven at 100°C. until free from moisture, and ashed. All ash percentages were calculated on the basis of extracted dry bone. The colorimetric method of Benedict and Theis (5) was used and the inorganic phosphorus was calculated to mg. of phosphorus per 100 cc. of serum.

## DISCUSSION.

Charts 1, 2, and 3 are self-explanatory. It will be noted in Chart 1 that the normal rats, on Ration A, grew unusually well, as might be expected on a ration of that type. It has been our experience that our rats are somewhat larger at 3 weeks of age than rats reared in some laboratories. This may be due to the unusually varied diet that we feed to our breeding colony.

While the rats receiving Ration B did not grow well, we were unable to observe any symptoms of rickets or other abnormalities. These rats appeared normal and remained active until the end of the experiment. The rats receiving Ration C, the rachitic ration,

began to show rachitic symptoms in about 2 weeks and none of them lived beyond the 7th week.

The inorganic blood phosphorus in the normal group, Ration A (Chart 2), tended to remain fairly constant at a level of about

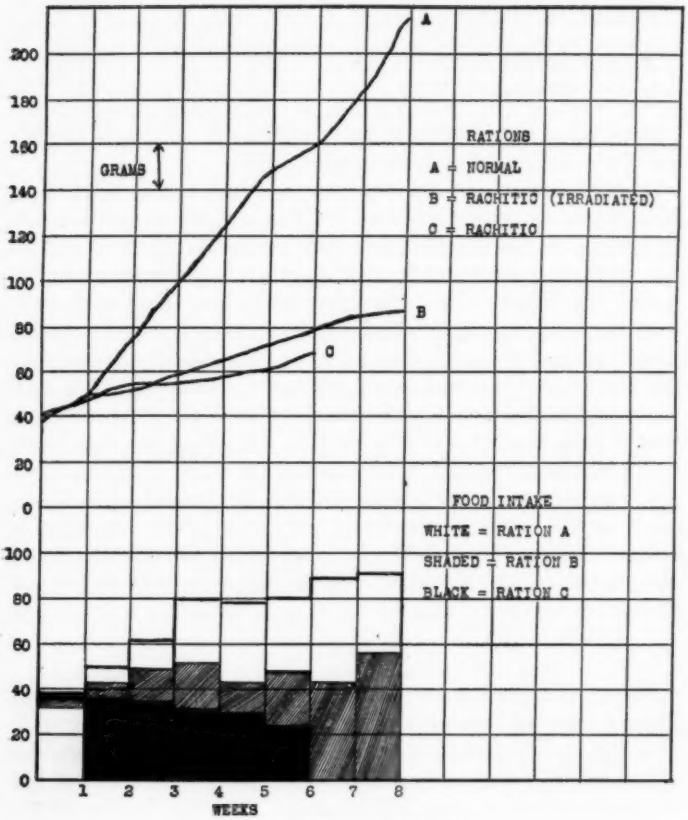


CHART 1. Curves of growth.

10 mg. per 100 cc. of serum. The rachitic bloods showed an immediate fall in inorganic phosphorus, which continued for about 3 weeks, when it rose slightly, due to the fact that the animals had gradually lost appetite, resulting in a lowered food

intake. This agrees with the observations of McCollum and coworkers (6) and Cavins (7) who found that fasting tended to cause an increase of inorganic blood phosphorus when the level of this blood constituent is relatively low. It is of interest to note that ultra-violet irradiation had the effect of causing the

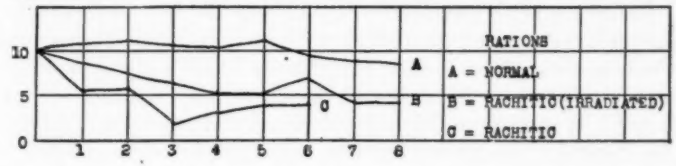


CHART 2. Inorganic blood phosphorus expressed in mg. per 100 cc. of serum.

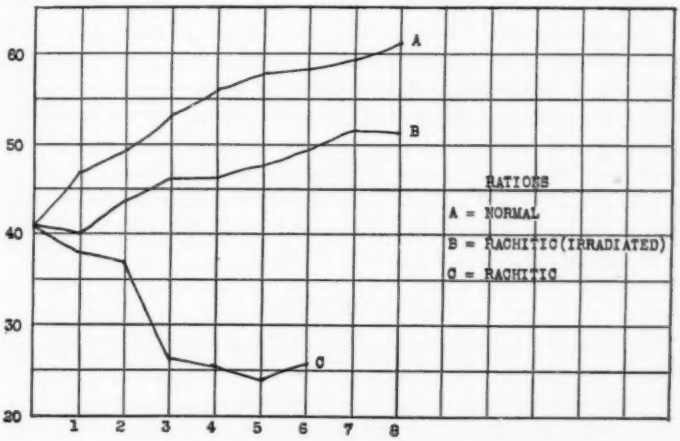


CHART 3. Bone ash expressed in per cent of dry, extracted femurs.

inorganic phosphorus to lie midway between the normal and the rachitic levels.

Examination of Chart 3 shows a similar situation with regard to the bone ash in the femurs. It is of importance to note that the percentage of ash rises steadily in the normal animals as age progresses. Our figures compare very favorably with those of Steenbock (4) in this regard. When the rachitic ration is irradiated there is no abrupt fall in bone ash; on the contrary, the tend-

ency seems to be for the ash to rise in a rather consistent manner. When the ration is not irradiated, however, the percentage of bone ash, in extracted bone, drops immediately and continues to do so until fasting affects the situation, which in our work did not occur until after the 3rd week.

Another point which merits emphasis is the fact that calcium and phosphorus deposition (Ration C) seems to be inhibited from the beginning of the feeding experiment. This leads us to raise the question as to the ability of the rat to store the antirachitic factor. If storage of this factor does occur we would not expect the blood phosphorus and the bone ash to fall, for a time, until the storage supply was depleted. This does not seem to be the case in our rats, however, and we are led to believe that the rat is not capable of storing significant amounts of the antirachitic factor.

It would appear, therefore, that these chemical methods should be very useful in making accurate estimations, in a relatively short time, of the antirachitic potency of our common foods. It offers a quantitative method of unusual interest, for we should be able to find, by feeding experiments, the minimum amount of any food which will prevent an appreciable fall of inorganic blood phosphorus or bone ash below the normal level of a normal 40 gm. rat or the minimum amount of any food which will bring about normal deposition of mineral matter in bones.

## CONCLUSIONS.

In normal rats, weighing between 35 and 40 gm., the inorganic blood phosphorus was 10 mg. per 100 cc. of serum and during an 8 week feeding period, the tendency was to maintain this level, falling slowly to 8 mg. per 100 cc. of serum at the end of 8 weeks. The percentage of ash in the dry extracted femurs of the same normal rats was 40 at the beginning of the experiment and increased steadily, during the 8 week period, to 62 per cent.

When rats received Steenbock's rachitic ration, inorganic blood phosphorus dropped from 10 mg. to 1.6 mg. in a period of 3 weeks. After that period it rose slightly, due to fasting. The ash of dry extracted femurs of the rachitic rats fell from 40 per cent to 26.5 per cent in 3 weeks and finally fell as low as 24 per cent.

When Steenbock's rachitic ration was irradiated with ultraviolet light the inorganic blood phosphorus and the percentage of bone ash fell midway between the normal and rachitic groups.

It would appear that the rat does not possess the ability to store the antirachitic factor in significant amounts for the reason that calcium and phosphorus deposition is hampered from the very beginning of the experimental feeding period.

The method as outlined offers an opportunity to make a quantitative study of the antirachitic properties of food materials, using as a standard the least amount of any food which will prevent fall in the percentage of bone ash, below that possessed by the young normal animals at the beginning of the experiment or the minimum quantity of food required to bring about optimal deposition of mineral matter. Work is now in progress with the view of attempting to define the conditions under which this method may be applied in the study of the antirachitic properties of food materials.

The writers wish to take this opportunity to acknowledge the generosity of Dr. Steenbock in allowing them to use his rachitic ration, prior to its publication by him.[1]

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[1] After this manuscript had been prepared, Dr. Steenbock published data concerning this ration (Steenbock, H., and Black, A., *J. Biol. Chem.*, 1925, lxiv, 263).

# THE DETERMINATION OF GASES IN BLOOD AND OTHER SOLUTIONS BY VACUUM EXTRACTION AND MANOMETRIC MEASUREMENT.

## III. GASOMETRIC DETERMINATION OF METHEMOGLOBIN.

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(Received for publication, September 21, 1925.)

Barcroft and Müller (1911) apparently proposed the first method for quantitative determination of methemoglobin. It was based on the fact that methemoglobin does not form a reversible compound with oxygen. Consequently the difference between the total hemoglobin (determined colorimetrically) and the portion of hemoglobin capable of combining with oxygen indicated the methemoglobin. Subsequent methods have been based on the same principles. The improvements have been in the methods for determining the total hemoglobin and the active hemoglobin, as we may call the hemoglobin capable of combining with oxygen.

In the recent literature three accurate methods have appeared.

Stadie (1920) changes both methemoglobin and active hemoglobin to cyanhemoglobin, and determines the total hemoglobin colorimetrically as cyanhemoglobin.

Nicloux (1924) by the simultaneous action of sodium hydrosulfite and carbon monoxide changes both methemoglobin and active hemoglobin to carboxyhemoglobin, which he estimates from the amount of CO held in combination. In accordance with Conant's elucidation of the relationship between methemoglobin and active hemoglobin, the reaction of methemoglobin may be schematically represented as

| | | | | | | |
|---|---|---|---|---|---|---|
| HbOH | + H | ⟶ | Hb | + CO | ⟶ | HbCO |
| Methemoglobin. | Reduction by hydrosulfite. | | Reduced hemoglobin. | | | Carboxy-hemoglobin. |

The inactive methemoglobin is reduced by hydrosulfite to reduced

hemoglobin, which is capable of combining with $O_2$ or CO. Nicloux accordingly estimated the methemoglobin from the difference in CO-combining capacity before and after treatment with hydrosulfite.

Conant and Fieser (1925) also reduce the methemoglobin to reduced hemoglobin. They make their analyses, however, by determining the oxygen-combining capacity, instead of the equivalent CO capacity. As reducing agent they found that they could not use hydrosulfite because some of its products catalyzed the regeneration of methemoglobin which began as soon as oxygen was admitted to the reduced solution. They obtained excellent results, however, when they performed the reduction with beta-anthrahydroquinone sulfonate, and limited the subsequent period of oxygenation to 10 or 15 seconds before drawing the sample for determination of combined oxygen. Even when beta-anthrahydroquinone sulfonate was used as reducing agent, longer exposure to oxygen resulted in formation of appreciable amounts of methemoglobin, and consequently low results for total hemoglobin estimated from the oxygen capacity.

As Conant and Fieser point out, their entirely gasometric technique has an advantage over the partially colorimetric method of Stadie, in that the presence of hematin or other colored products does not interfere with the former.

Nicloux's carbon monoxide gasometric method has the same advantages. It also has an advantage over the technique of Conant and Fieser in that the possibility of regeneration of methemoglobin is avoided by the use of CO instead of $O_2$ for saturation of the reduced hemoglobin.

In the technique described in the present paper, we have adapted Nicloux's method to use with the manometric blood gas apparatus, with which rapid and accurate CO determinations have been described by Van Slyke and Neill (1924), and by Harrington and Van Slyke (1924).

### *Method for Determination of Methemoglobin.*

The reagents used are the following:

*Acid Ferricyanide Solution.*—Described by Van Slyke and Neill.[1]

[1] Van Slyke and Neill (1924), p. 563.

*Air-Free 1 N Sodium Hydroxide Solution.*—Described by Van Slyke and Neill.[2]

*Carbon Monoxide Gas.*—This may be prepared by dropping concentrated sulfuric acid upon crystals of oxalic acid or into anhydrous formic acid, and warming with a burner, the evolved gas being collected in a gasometer. Oxalic acid yields per gm. about 275 cc. each of $CO_2$ and CO; the gases must accordingly be washed with alkali to remove $CO_2$. Formic acid yields per cc. about 500 cc. of pure CO.

*Nicloux's Ammoniacal Solution of Sodium Hydrosulfite.*—Place in a 100 cc. beaker 2 gm. of pulverized $Na_2S_2O_4$. Mix in another beaker 50 cc. of water and 1 cc. of concentrated ammonia solution. Pour the dilute ammonia solution onto the hydrosulfite, and cover the solution at once with a layer of paraffin oil about 1 cm. deep to prevent oxidation by air. Dissolve the hydrosulfite by stirring for a few seconds with a rod. Commercial hydrosulfite usually contains some insoluble impurity; consequently one does not attempt to stir until complete solution is attained.

*Procedure of Analysis.*—Place in a separatory funnel, preferably of cylindrical rather than conical shape, either 5 or 10 cc. of blood, accurately measured, and $\frac{1}{5}$ as great a volume, also accuraetly measured, of the freshly prepared ammoniacal hydrosulfite solution, making blood solution A. Smaller amounts of blood may be used if micro determinations of the CO are made. The separatory funnel should be large enough so that the blood will spread over the walls in a thin layer when rotated. A 100 cc. funnel is convenient for 5 cc. of blood, which is used when 1 cc. samples are to be taken for CO determination.

The air in the funnel is immediately replaced by CO at atmospheric pressure. This may be accomplished by passing through the funnel several times the volume of CO that would fill it. Somewhat more economically, with respect to CO consumption, it may be accomplished by alternately evacuating the funnel and refilling it with CO three times.

In a second similar funnel are placed the same volume of blood, $\frac{1}{5}$ volume of water (instead of hydrosulfite solution), and an atmosphere of CO, in the same manner (blood solution B).

The vessels are now rotated, either by hand or by a mechanical rotator like that of Stadie (1921), for 15 minutes or more. Then samples of 0.2, 1.0, or 2.0 cc., according to the amount of blood available, are drawn from the funnel into pipettes and used for

[2] Van Slyke and Neill (1924), p. 534.

carbon monoxide determinations. It does not matter how long the blood is left in the vessels with the CO atmosphere, but the samples for analysis should be drawn into pipettes, especially in the case of the blood treated with hydrosulfite, as soon as the funnel is opened to the air.

The carbon monoxide determinations are carried out as described by Van Slyke and Neill,[3] with the simplification that absorption of $O_2$ from the extracted blood gases is here unnecessary, since all the $O_2$ has been replaced by CO. The $CO_2$ is absorbed with the 1 N air-free sodium hydroxide, and $p_1$ is measured on the manometer. The gas is then ejected from the apparatus, and $p_2$ is measured. The CO pressure is

$$P_{CO} = p_1 - p_2.$$

*Calculation.*—The carbon monoxide contents are calculated by means of Table II or III of Van Slyke and Neill. In order to correct for the dilution with $\frac{1}{5}$ volume of hydrosulfite solution or water each result is multiplied by $\frac{6}{5}$. The methemoglobin is calculated as follows, in terms of $O_2$ or CO capacity:

$$\text{Methemoglobin} = (\text{CO in A}) - (\text{CO in B}).$$

The total hemoglobin content may be estimated as

$$\text{Total Hb} = (\text{total CO in A}) - (\text{dissolved CO}).$$

The hemoglobin values are thus obtained in terms of volume per cent or millimols per liter of oxygen (or CO) capacity.

The estimation of the "dissolved CO" is somewhat uncertain because the solubility coefficient of CO in blood has not been accurately ascertained by direct determinations. These were attempted by Hüfner (1894), but satisfactory results were prevented by the difficulty in differentiating between physically dissolved and combined CO. However, we have found that sufficiently close results are obtained if the solubility is assumed to be proportional to the water content of the diluted blood, or 85 per cent of the solubility in pure water. Thus estimated the dissolved CO taken up when the saturation is performed at a barometric pressure of 760 mm. is in terms of volumes per cent,

[3] Van Slyke and Neill (1924), p. 562.

2.16 at 15°, 1.95 at 20°, 1.82 at 25°, and 1.71 at 30°. The values in terms of millimolar concentrations are 0.99 at 15°, 0.87 at 20°, 0.8 at 25°, and 0.76 at 30°. Unlike the calculation of total hemoglobin, that of methemoglobin does not involve a correction for physically dissolved CO. The physically dissolved CO is practically the same in both blood samples, A and B, saturated with the gas under the same conditions, and hence does not affect the difference between them, due to combined CO.

*Substitution of Illuminating Gas for Pure Carbon Monoxide.*—The common type of illuminating gas is about one-third CO, and we find that the New York gas may be used in place of CO without significantly decreasing the accuracy of the methemoglobin estimation.

If total hemoglobin is to be estimated, however, the solubility of the gas must be determined by saturating about 15 cc. of water with the gas in another separatory funnel, and determining the gas content of the saturated water. 5 cc. of the saturated water are transferred to the blood gas apparatus and the gases are extracted without addition of reagents. The extracted gases are treated with 1 N sodium hydroxide solution to remove any $CO_2$ which may be present, and are then measured by reading the pressure before and after ejection from the apparatus, as in the above described CO determination. The gas content of the water is multiplied by 0.85 to estimate the amount dissolved under the same conditions in blood.

The solubility coefficient of illuminating gas is likely to be twice that of CO, and probably the results for total hemoglobin obtained with it are subject to somewhat more error than when pure CO is used. We have not found significant differences in the content determinations we have carried out, but illuminating gas made from coal varies in its composition, and whether all types of gas will yield good total hemoglobin figures one cannot predict.

Results obtained with the method above described are given in the accompanying paper by Van Slyke and Vollmund.

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# STUDIES OF METHEMOGLOBIN FORMATION.

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(Received for publication, September 21, 1925.)

Conant (1923) and Conant and Fieser (1925) have shown that it is characteristic of methemoglobin that by proper reducing agents it is transformed into reduced hemoglobin, with restoration of ability to combine with oxygen and carbon monoxide. The ability to be changed by reduction from a substance incapable of binding molecular $O_2$ or CO into reduced hemoglobin appears to be the most specific chemical characteristic of methemoglobin that we know. Determinations of methemoglobin based on it may therefore be considered to be more certain to measure that substance, and exclude other hemoglobin derivatives, than may qualitative spectroscopic examinations or quantitative estimation of loss of oxygen capacity, uncontrolled by reduction to reduced hemoglobin. The methods of Conant and Fieser (1925), of Nicloux and Fontès (1924), and the modification of the latter in the preceding paper by Van Slyke (1925), include this control. It appears desirable to review experimentally with such methods the effects on hemoglobin of some of the substances that have been considered to transform hemoglobin into methemologbin.

We have, accordingly, with the technique described in the preceding paper, studied the effects on hemoglobin of aniline, nitrobenzene, ferricyanide, and nitrite, which represent four different types of supposed methemoglobin formers.

## *Aniline.*

### *Time Reaction of Aniline and Hemoglobin in Blood.*

To 100 cc. of horse blood containing 11.3 millimols of hemoglobin per liter (by CO capacity) 4 mols of aniline were added per mol of hemoglobin (the amount of aniline calculated for 4 mols per mol of Hb is 93 × 11.3

$\times \frac{160}{1000} = 104$ mg. The amount added was 0.10 cc.) The aniline and blood were mixed and agitated in a water bath in cylinders filled with air at 38°. A similar experiment was performed with half as much aniline. At varying periods samples were withdrawn and used for determination of total hemoglobin and methemoglobin as described by Van Slyke (1925). The blood was not hemolyzed to a significant extent. The results are indicated in Fig. 1.

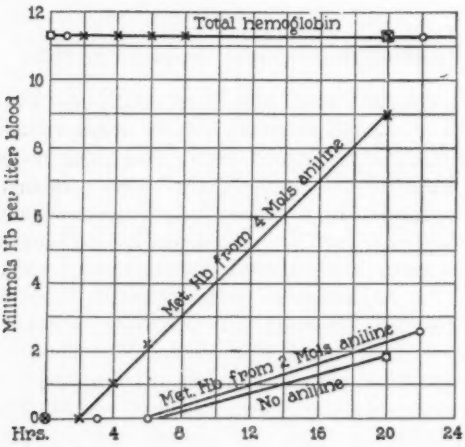


FIG. 1. Rate of formation of methemoglobin by aniline at 38°.

It is evident that the kinetics of methemoglobin formation by aniline are not simple. The outstanding qualitative facts are that:

(1) The reaction is a slow process. It is increased in rate by increasing aniline concentrations. The quantitive relationship between aniline concentration and rate of methemoglobin formation does not appear to be one of simple direct proportion, as is evidenced also by the data in Fig. 2.

(2) The reaction has a peculiar lag. No methemoglobin at all was formed by 4 mols of aniline at 38° until after 2 hours, and none by 2 mols until after 5 hours. The lag is even more marked if the reaction is carried out at room temperature, as shown by Table I.

In the experiment there recorded varying amounts of aniline were mixed with oxygenated blood and permitted to stand at

room temperature. (The total hemoglobin contents in the different samples vary somewhat, apparently because of sedimentation of corpuscles in the stock of oxalated horse blood from which the material was taken.)

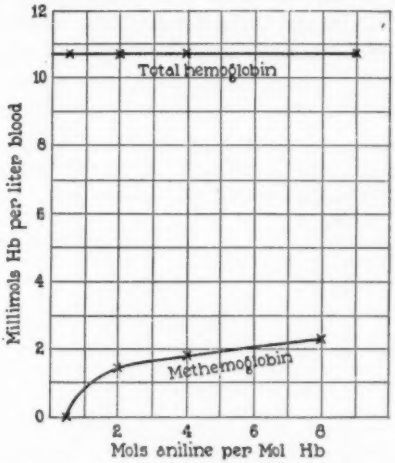


FIG. 2. Effect of variation in aniline concentration on methemoglobin formation at 25°.

TABLE I.

*Lag in Formation of Methemoglobin by Aniline at 25°.*

| Mols aniline per mol Hb. | Time of reaction. | Total Hb. | Active Hb. | Methemoglobin. |
|---|---|---|---|---|
| | *hrs.* | *mM* | *mM* | *mM* |
| 0.5 | 2 | 9.5 | 9.5 | 0.0 |
| 2.0 | 2 | 9.3 | 9.3 | 0.0 |
| 4.0 | 4 | 9.5 | 9.5 | 0.0 |
| 8.0 | 4 | 11.3 | 11.3 | 0.0 |
| 8.0 | 16 | 8.8 | 6.4 | 2.4 |

The lag suggests that the methemoglobin formation is caused not by the aniline itself, but by some product into which it is changed by the blood. Ellinger (1920) obtained evidence that acetanilide *in vivo* is converted into acetyl-phenyl-hydroxylamine by change of the $C_6H_5$-$\dot{N}H$ group to $C_6H_5$-$\dot{N}(OH)$. It is possible

that aniline is changed similarly. From the chemical structure of aniline itself it would be difficult to imagine how it would act either as an oxidizing agent or a "hydrogen acceptor." Heubner and Rohde (1923) found that phenyl-hydroxylamine is in fact a former of methemoglobin. Lipschitz and Weber (1924) found that $C_6H_5NH_2$ and $C_6H_5NH(OH)$ have no effect on reduced hemoglobin, but form methemoglobin in the presence of oxygen.

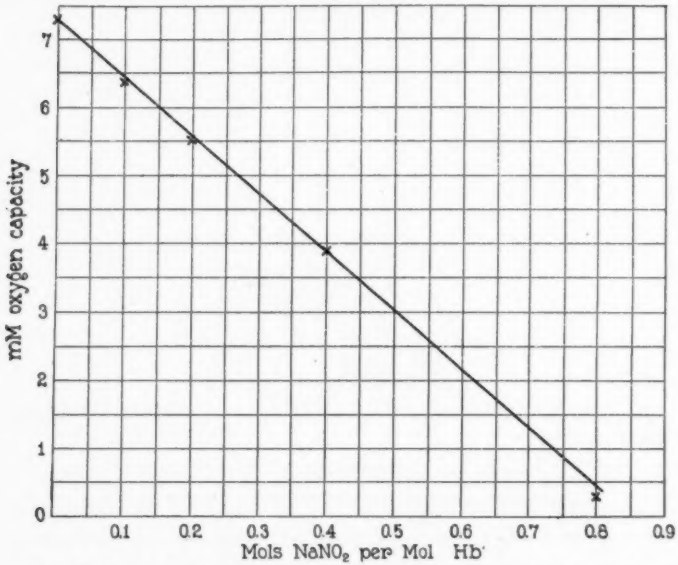


FIG. 3. Approximate formation of 1 mol of methemoglobin per 1 mol of nitrite at 25°.

A possible explanation for the formation of methemoglobin in blood by aniline is that the latter is converted into phenylhydroxylamine, which acts as a catalyzer to accelerate the oxidation of reduced hemoglobin by molecular oxygen to methemoglobin. The scope of the present work has prevented further testing of this hypothesis.

We have attempted to produce methemoglobin formation in rabbits *in vivo* by painting the shaven abdomen with aniline to simulate the conditions under which human aniline intoxication

usually happens. As in Stadie's (1921) attempts to produce methemoglobinemia in rabbits, however, the result was only anemia. Presumably methemoglobin is formed, but does not stay in the blood. The results of an experiment, in which the painting was repeated daily for 4 days, are given in Table II. The skin at the end of the experiment was necrotic.

That the aniline penetrates the blood cells and forms methemoglobin within them *in vitro* is indicated by the following experiment.

Horse blood was mixed with 2 mols of aniline per mol of hemoglobin, and left overnight at room temperature. The cells were than centrifuged and washed several times. The saline suspension was dark brown and gave the following CO capacities:

After treatment with $Na_2S_2O_4$.....6.62 mM per liter.
Without " " " .....4.48 " " "
Methemoglobin.....2.14 " " "

TABLE II.

*Result of Painting Rabbit's Abdomen 4 Days with Aniline.*

| | Total Hb. | Active Hb. | Met. Hb. |
|---|---|---|---|
| | *mM* | *mM* | *mM* |
| Before painting..... | 8.2 | 8.2 | 0.0 |
| After " ..... | 5.5 | 5.5 | 0.0 |

The fact that aniline *in vivo* causes a disappearance of hemoglobin (presumably after transformation to methemoglobin) instead of the appearance of methemoglobin indicates an effect of factors, presumably hemolytic, which do not act *in vitro*.

The above results demonstrate (1) that the product formed from the action of aniline on hemoglobin is genuine methemoglobin, so far as we can characterize it at present, (2) that the process of formation is a slow one, with a peculiar latent period during which no formation at all occurs, and (3) that the process can occur in the cells, which therefore appear permeable either to aniline or to whatever product of it produces the change to methemoglobin.

*Nitrobenzene.*

Nitrobenzene is one of the substances usually listed as methemoglobin formers. It is certain that it can reduce the oxygen-

combining capacity of blood *in vivo*, but whether the inactive substance into which the hemoglobin is changed is methemoglobin or some other derivative appears less certain. Loeb, Bock, and Fitz (1921) in two men with nitrobenzene poisoning found the oxygen capacity of the blood reduced to 6.2 and 8.9 volumes per cent respectively. The red cell count and total hemoglobin by Stadie's method were normal, corresponding to about 20 volumes per cent of oxygen capacity. The differences between total hemoglobin and oxygen capacity would indicate that over half the hemoglobin was inactivated in each case. Yet no methemoglobin could be detected by the spectroscope.

We have attempted to simulate human nitrobenzene poisoning in rabbits by painting the skin with the substance, and by administering it with a stomach tube. The effect seems quite

TABLE III.

*Effect of Nitrobenzene on Carbon Monoxide Capacity of Blood Determined with and without Hydrosulfite.*

| Blood. | Nitrobenzine. | Approximate mols nitrobenzene per mol Hb. | CO capacity of blood. | | |
|---|---|---|---|---|---|
| | | | Without nitrobenzene or hydrosulfite. | With nitrobenzine without hydrosulfite. | With nitrobenzene and hydrosulfite. |
| *cc.* | *cc.* | | *mM* | *mM* | *mM* |
| 5 | 0.05 | 10 | 8.4 | 8.3 | 7.9 |
| 10 | 0.006 | 0.5 | 9.6 | 9.5 | 8.9 |

different from that in man. Relatively much more severe treatment appears required to produce intoxication in the rabbit, and the effect on the blood is to produce not inactive hemoglobin or methemoglobin, but anemia. Thus, an animal killed by 5 days painting of the abdomen showed at death the oxygen capacity reduced to 4.5 volumes per cent, but there was no evidence of methemoglobin. The spleen was filled with dark pigment.

If nitrobenzene and blood are mixed *in vitro* the mixture exhibits a peculiar behavior. The nitrobenzene produces no immediate decrease in the CO-combining capacity of the blood untreated with hydrosulfite. When the blood is treated with hydrosulfite, however, an actual decrease occurs in the CO capacity, as though the hydrosulfite, instead of changing methemoglobin back to

reduced hemoglobin, had the reverse effect (see Table III). It is evident that the hydrosulfite-CO methemoglobin method is not suitable for determination of the product formed by the action of nitrobenzene on hemoglobin.

*Ferricyanide.*

Potassium ferricyanide, as shown by common experience in blood oxygen determinations, and by Conant's (1923) electrometric titrations, reacts almost instantly with dissolved hemoglobin to form methemoglobin. The ferricyanide seems quite unable to penetrate the cells, however. Even after some hours standing with equimolar amounts of ferricyanide we have found blood with intact cells to show but a trace of methemoglobin formation. The presence of ferricyanide in the amounts used did not interfere with the hydrosulfite-CO method.

The following experiment demonstrates the impermeability of the cells to ferricyanide.

10 cc. of a suspension of horse erythrocytes in 0.9 per cent NaCl solution (hemoglobin content = 4.7 mM) were treated with 0.5 mol of potassium ferricyanide per mol of hemoglobin and kept 4 hours at 38°. The cells were washed several times with saline solution, and were finally suspended in saline solution and analyzed for methemoglobin.

| | *mM* |
|---|---|
| CO capacity after hydrosulfite | 3.72 |
| " " without " | 3.76 |
| Methemoglobin | 0.0 |

As a control, blood hemolyzed with saponin was treated with 0.5 mol of ferricyanide per mol of hemoglobin.

| | *mM* |
|---|---|
| CO capacity after hydrosulfite | 11.32 |
| " " without " | 4.53 |
| Methemoglobin | 6.79 |

Stadie (1921) found that relatively large amounts of ferricyanide were necessary to cause disappearance of hemoglobin from rabbit blood, and that the rate of disappearance was much slower than after nitrite injection. Presumably the impermeability of the cells to ferricyanide is responsible for the difference. It may also be responsible for the relatively slight toxicity of ferricyanide administered orally. It is furthermore the impermeability of the cells for ferricyanide that necessitates complete laking of blood

before the oxygen content can be determined by the ferricyanide method.

*Nitrites.*

Nitrites, like ferricyanide, quickly change active hemoglobin to a form which does not bind $O_2$ or CO. That nitrite hemoglobin is the same methomoglobin as that formed by the action of ferricyanide or spontaneous oxidation of partially oxygenated blood appears uncertain from the results of Hartridge (1920). The product does, nevertheless, react with hydrosulfite like methemoglobin, with regeneration of reduced hemoglobin.

10 cc. of a cell solution containing 4.3 mM of Hb by oxygen capacity measurement were treated with 10 mg. of $NaNO_2$ (3 mols per 1 mol Hb). After a few minutes the solution was divided into two parts, and the CO capacities were determined with and without hydrosulfite, as described for methemoglobin determination.

| | mM |
|---|---|
| CO capacity with hydrosulfite | 4.5 |
| " " without " | 0.4 |
| Methemoglobin | 4.1 |

Unlike ferricyanide, nitrite penetrates the red cells, as shown by the following.

A suspension of washed horse cells in saline had oxygen capacity of 8.1 mM. Two portions were treated, one with 5 mols of potassium ferricyanide per mol of hemoglobin, the other with 5 mols of sodium nitrite. After 30 minutes both suspensions were centrifugated, washed, made up to original volume, and the oxygen capacities were redetermined.

| | mM |
|---|---|
| Suspension treated with nitrite, | $O_2$ capacity = 0.89 |
| " " " ferricyanide, | $O_2$ " = 8.15 |

The rapidity of the action of nitrite is indicated by the following experiment.

A hemoglobin solution was prepared by laking 5 cc. of horse erythrocytes in 10 cc. of water. The oxygen capacity of the solution was 6.70 mM. To 10 cc. of the solution 5 mg. of $NaNO_2$ were added (1.07 mols nitrite per mol hemoglobin) and the oxygen capacities were determined at intervals. (See Table IV.)

The fact that approximately 1 mol of nitrite reacts with 1 mol of hemoglobin is shown by the following experiment.

A solution of cells containing 7.30 mM of hemoglobin, by oxygen capacity measurement, was divided into 5 cc. portions. To four such portions were added respectively 0.05, 0.1, 0.2, and 0.4 cc. portions of 1 per cent $NaNO_2$ solution, containing 0.1, 0.2, 0.4, and 0.8 mols of nitrite per mol of hemoglobin. (The $NaNO_2$ stock solution was standardized by permanganate titration.) After 30 minutes at room temperature the oxygen capacities were redetermined. The results are given in Fig. 3.

The permeability of the cells for nitrite is presumably the cause for the facts noted by Stadie (1921), that the effect of nitrite on the blood of rabbits *in vivo* occurs in a few minutes, that intravenously injected nitrite causes the formation of about equimolar amounts of methemoglobin, and that the methemoglobin remains in the cells.

TABLE IV.

*Rate of Reaction of Nitrite and Hemoglobin.*

| Time after addition of nitrite. | Oxygen capacity. |
|---|---|
| *min.* | *mM* |
| 0 | 6.7 |
| 5 | 1.5 |
| 20 | 0.4 |
| 40 | 0.4 |
| 60 | 0.4 |

## SUMMARY.

Nitrobenzene acting on blood *in vitro* did not yield a product determinable as methemoglobin by the hydrosulfite-CO method. When absorbed by rabbits nitrobenzene caused anemia, without methemoglobinemia.

The actions of aniline, ferricyanide, and nitrite on hemoglobin in the presence of air yielded products which were identical with methemoglobin in that they could not bind molecular $O_2$ or CO until converted by reduction with hydrosulfite into reduced hemoglobin.

The action of aniline showed a latent period at the beginning, no methemoglobin being formed for a time that might extend for some hours. After methemoglobin formation began it proceeded slowly, and several mols of aniline per mol of hemoglobin were required to complete it. The behavior accords with the possi-

bility, indicated by previous writers, that a product of aniline rather than aniline itself causes the methemoglobin formation. Aniline forms methemoglobin in the cells of unlaked blood: consequently either aniline or its product penetrates the cells.

Nitrite and ferricyanide alike react almost instantly in laked blood to form methemoglobin, and 1 mol of each forms approximately 1 mol of methemoglobin.

Nitrite and ferricyanide differ in that nitrite penetrates the cells instantly and forms methemoglobin within them. The cells, on the contrary, appear entirely impermeable to ferricyanide anion, and methemoglobin formation by ferricyanide *in vitro* occurs only after they are laked.

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# DIETARY FACTORS INFLUENCING CALCIUM ASSIMILATION.

## VI. THE ANTIRACHITIC PROPERTIES OF HAYS AS RELATED TO CLIMATIC CONDITIONS WITH SOME OBSERVATIONS ON THE EFFECT OF IRRADIATION WITH ULTRA-VIOLET LIGHT.*

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Plate 1.

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As early as 1913 the observation was made in this laboratory by Steenbock and Hart (1) that the assimilation of lime by the goat was greatly improved after the animal had received green grasses and had been out of doors as compared with an earlier period when the animal was kept indoors on a ration of straws and grains. Since then many experiments involving the determination of calcium balances have been conducted with both goats and cows receiving legume hays. These experiments were conducted primarily for the purpose of determining whether such legume roughages, comparatively rich in calcium, could maintain positive calcium balances in liberally milking animals. In one experiment conducted in 1922 (2) we were able to maintain heavily producing milch cows in positive calcium balance when there was a daily allowance of 10 pounds of alfalfa hay in a ration made up of corn silage and grains. This alfalfa hay had been "cured under caps."

In another experiment with milking cows, reported later (3),

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we again used alfalfa hay in proportions similar to those used in our first experiment; but here we did not secure a positive calcium balance. In fact, a negative calcium balance resulted. These later data, where negative calcium balances were secured, were in agreement with most of the results obtained by other experimenters (4) in this field when using legume hays as a roughage in the ration of dairy cows. In this second experiment the alfalfa hay had been "cured in the windrow" for 4 days with an exposure to air and light.

It appeared to us at that time that the differences in the results obtained could be explained by assuming a greater destruction of the factor concerned with calcium assimilation in the hay cured in the windrow with exposure to air and light than in the case of the hay cured under caps. We thought of this factor as something originating in the plant through its processes of metabolism as is the case with vitamins A, B, and C and that it probably suffered greatest destruction with a maximum exposure to air and light. Here we were influenced by our findings in studies designed to test the stability of vitamin A, which showed that alfalfa hay cured under a skylight in our attic and allowed to remain there for many months until completely bleached no longer possessed any growth-promoting properties for rats. Except for being cured under caps we had no further knowledge of the hay used in our first experiment where positive calcium balances were obtained. We knew nothing whatsoever about the time of its actual exposure to sunlight.

Since these earlier experiments we have cured many legume hays in various ways involving long exposure to weather (rain, dew, sunshine, etc.) and have also cured them in our dark attic with a minimum exposure to the weather. These hays have been used in calcium metabolism experiments with dry and milking goats in attempts to determine under what conditions of curing a maximum antirachitic potency could be obtained. This point is of very great practical importance, especially for the feeding of farm animals during the winter months. The results secured were conflicting and no generalizations seemed possible from the body of data secured. In some cases the animals (milking goats) were held in positive calcium balance; in other cases a negative balance prevailed, and without any

consistent correlation with the curing processes. It was apparent that the antirachitic property of these legume hays was feeble; at least when compared with such potent agents as cod liver oil.

In the summer of 1924 we again cured hays (this time from green clover) under varying conditions with the hope of throwing more light upon this problem. The clover used represented the first cutting of the season and consisted of red clover mixed with some alsike clover. It was in the stage of growth where blossoms were beginning to appear. It was of excellent quality and was cut in the morning of June 26. It was divided into three portions designated respectively 1, 2, and 3.

No. 1 was dried in the dark on the floor of our attic before a fan. Here it was allowed to remain for several weeks. It was a product of fine green color.

No. 2 was dried in the diffused light of our laboratory and then exposed to the weather for 14 days, but brought inside at night and during cloudy weather when rain threatened. It was abundantly exposed to sunlight, but no actual record of the time of exposure was kept. The product was of a brownish green color.

No. 3 was allowed to lie in the field exposed to sun, dew, and rain for a period of 14 days. During this time it was actually rained upon six different times. The product had the appearance of a ripe clover straw.

All samples were cut up with a feed cutter and stored in the dark in bags until fed.

During the progress of these investigations the idea was being experimentally developed in this laboratory by Steenbock and coworkers (5) and by Hess (6) that antirachitic properties can be imparted to plant and animal materials by exposing them directly to ultra-violet light from various sources such as that from the quartz mercury vapor arc, the iron arc, the carbon arc, or from sunlight. According to this idea the antirachitic potency of a hay would depend upon the degree of its exposure to direct sunlight minus such destruction of the activity as might take place during the curing process. Upon these general premises investigation of the calcifying potency of the hays cured in the several ways stated above was carried out. Their anti-

rachitic activity was measured not only by the use of the rat but also with milking goats and growing chickens.

*Rat Experiments.*

Our experiments with rats were carried out by feeding the clover at levels of 1 and 5 per cent in a rickets-producing ration and then examining the animals for the severity of the rickets produced. To make the results as comparable as possible each sample of clover was fed to one rat from each litter and in addition irradiated samples of clovers were fed to other members of the same litter. These latter experiments allowed us to establish the degree to which the clovers—if active—could be activated under conditions which we know are favorable for the induction of antirachitic properties.

The samples of clover in preparation for feeding were first chopped in a feed cutter and then ground in an Excelsior Mill and finally in an Abbé Ball Mill to an impalpable powder. When irradiated they were exposed to the light from a Cooper-Hewitt BY type quartz mercury vapor lamp, the burner of which received a current of 50 volts and 4 amperes. Exposure was made at a distance of 2 feet for 30 minutes with the clover spread out in a very thin layer. It was stirred once during the operation.

The rickets-producing ration known as Ration 2965 was one devised in this laboratory, consisting of yellow corn 76, wheat gluten 20, calcium carbonate 3, and sodium chloride 1. In our hands it has given most excellent results producing uniformly incipient rickets in rats of our breeding in a few weeks and very severe rickets in 4 to 5 weeks. In this ration the clover was incorporated at the expense of an equal amount of yellow corn.

The rats used were raised in our laboratory under standardized conditions as described in previous publications. These were taken at an age of 24 to 27 days, weighing from 47 to 68 gm. After 32 to 40 days on the rations in two separate series note was taken of the severity of the rachitic condition in each animal as indicated by enlargement of the joints—particularly the wrists—and by general behavior. Invariably this diagnosis was confirmed by later gross examination of the costochondral junctions and by silver staining of the distal ends of the radii and ulnæ after splitting with a scalpel. In one series of experiments where

the clover was fed at a 1 per cent level the femurs were ashed after exhaustive extraction of their lipoids with hot alcohol.

The results of the rat experiments are summarized in Tables I and II. At a 5 per cent level of intake it is seen that Hay 1, cured in the dark, was ineffective in preventing the rachitic condition. Hay 2, cured in the sun, prevented rickets completely and Hay 3, cured in the sun, dew, and rain, was partially protective. The positive, though somewhat reduced antirachitic action of Hay 3, as compared with Hay 2, was an unexpected result because we had surmised that its long continued exposure to sun, dew, and rain, which had bleached it until it looked like clover straw, would have left little of the properties associated with the presence of the antirachitic vitamin. Since these observations, however, we have made other observations showing that the antirachitic action resident in irradiated food materials is a rather stable property. It may, however, be destroyed permanently under some conditions, but under others it is merely inactivated—again becoming active upon reexposure to ultra-violet radiations. These relations will be discussed in a subsequent paper. The table further shows that in all cases where the hays were irradiated the bones of all animals were apparently normal as far as gross observations could reveal. In these instances the behavior of the animals was also normal. Plate 1 presents photographic evidence of the enlargement of the costochondral junctions of Rat 2445 receiving 5 per cent of the hay cured in the attic. In contrast with this there is presented a picture of the normal costochondral junctions of Rat 2449 which received 5 per cent of the same hay after it had been irradiated with ultra-violet light.

When the hays were fed at a 1 per cent level the results were of a slightly different nature (Table II). 1 per cent of hay apparently did not furnish a sufficiency of the antirachitic factor in any case whether irradiated or not to prevent all indications of insufficiency. In general the irradiated hays showed themselves most potent. Next in order was Hay 2 cured in the sun; then Hay 3 cured in the sun, dew, and rain; and, least of all, Hay 1 cured in the dark. The latter, in fact, gave no evidence of antirachitic action which harmonized with what we observed when it was fed at a 5 per cent level.

TABLE I.

*The Effect of Feeding 5 Per Cent of Clover Cured in Various Ways When Incorporated in Rickets-Producing Ration 2965.*

| Lot. | Rat No. | Sex. | Initial weight. | Final weight. | Metaphyses. | Costochondral junctions. |
|---|---|---|---|---|---|---|
| | | | *gm.* | *gm.* | | |
| 3189. Hay 1, cured in dark. | 2445 | ♀ | 47 | 97 | Very wide. Decidedly rachitic. | Decidedly enlarged. |
| | 2446 | ♂ | 57 | 122 | | |
| | 2447 | ♀ | 51 | 105 | | |
| | 2448 | ♀ | 54 | 99 | | |
| 3190. Same but irradiated. | 2449 | ♀ | 65 | 130 | A narrow line. Normal. | Normal. |
| | 2450 | ♂ | 55 | 105 | | |
| | 2451 | ♀ | 51 | 98 | | |
| | 2452 | ♀ | 53 | 102 | | |
| 3191. Hay 2, cured in sun. | 2453 | ♀ | 68 | 129 | A narrow line. Normal. | Normal. |
| | 2454 | ♀ | 54 | 112 | | |
| | 2455 | ♂ | 50 | 123 | | |
| | 2456 | ♀ | 51 | 95 | | |
| 3192. Same but irradiated. | 2457 | ♀ | 57 | 109 | A narrow line. Normal. | Normal. |
| | 2458 | ♀ | 55 | 92 | | |
| | 2459 | ♂ | 52 | 92 | | |
| | 2460 | ♀ | 52 | 99 | | |
| 3193. Hay 3, cured in sun, dew, and rain. | 2461 | ♀ | 57 | 126 | About two-fifths as wide as in Lot 3189 except in Rat 2462 which was one-third as wide. Rachitic. | Slightly enlarged, especially in Rats 2462 and 2464. |
| | 2462 | ♀ | 52 | 99 | | |
| | 2463 | ♀ | 53 | 107 | | |
| | 2464 | ♂ | 54 | 147 | | |
| 3194. Same but irradiated. | 2465 | ♂ | 62 | 125 | A narrow line. Normal. | Normal. |
| | 2466 | ♀ | 56 | 85 | | |
| | 2467 | ♂ | 50 | 94 | | |
| | 2468 | ♂ | 52 | 100 | | |

TABLE II.

*The Effect of Feeding*

| | | | | |
|---|---|---|---|---|
| 2467 | ♂ | 50 | 94 | |
| 2468 | ♂ | 52 | 100 | |

TABLE II.

*The Effect of Feeding 1 Per Cent of Clover Cured in Various Ways When Incorporated in Rickets-Producing Ration 2965.*

| Lot. | Rat No. | Sex. | Initial weight. | Final weight. | Ash in femurs. | Metaphyses. | Costochrondral junctions. |
|---|---|---|---|---|---|---|---|
| | | | *gm.* | *gm.* | *per cent* | | |
| 3363. Hay 1, cured in dark. | 3334 | ♂ | 60 | 82 | 25.14 | Rachitic, about 2 mm. wide. | Rachitic, enlarged, and angulated. |
| | 3335 | ♀ | 62 | 97 | 23.92 | | |
| | 3336 | ♂ | 62 | 88 | 24.75 | | |
| | | | | | Average 24.60 | | |
| 3364. Same but irradiated. | 3337 | ♀ | 55 | 90 | 41.16 | Rachitic, about 0.5–1 mm. wide. | Normal. |
| | 3338 | ♂ | 65 | 132 | 42.99 | | |
| | 3339 | ♂ | 62 | 117 | 41.63 | | |
| | | | | | Average 41.92 | | |
| 3365. Hay 2, cured in sun. | 3340 | ♂ | 58 | 89 | 26.96 | Rachitic, about 1–1.5 mm. wide. | Rachitic, enlarged, not angulated. |
| | 3341 | ♂ | 65 | 127 | 33.88 | | |
| | 3342 | ♀ | 58 | 90 | 27 56 | | |
| | | | | | Average 29.46 | | |
| 3366. Same but irradiated. | 3343 | ♂ | 58 | 96 | 41.12 | Rachitic, about 0.5 mm. wide. | Normal. |
| | 3344 | ♂ | 62 | 105 | 43.32 | | |
| | 3345 | ♀ | 60 | | | | |
| | | | | | Average 42.22 | | |
| 3367. Hay 3, cured in sun, dew, and rain. | 3346 | ♀ | 56 | 94 | 31.53 | Rachitic, about 2 mm. wide. | Rachitic, enlarged, not angulated. |
| | 3347 | ♂ | 62 | 102 | 27.38 | | |
| | 3348 | ♀ | 50 | 80 | 30.61 | | |
| | | | | | Average 29.84 | | |
| 3368. Same but irradiated. | 3349 | ♂ | 56 | 93 | 41.37 | Rachitic, about 0.5–1 mm. wide. | Normal. |
| | 3350 | ♂ | 62 | 105 | 41.05 | | |
| | 3351 | ♂ | 58 | 86 | 42.02 | | |
| | | | | | Average 41.48 | | |

The table reveals considerable variations in the degree of abnormality of the rats depending upon what is used as a criterion. It is a well known fact that, in the human, different parts of the skeleton give evidence of the progressive development of rickets in a rather regular sequence. Thus Moore states that in the infant rickets will manifest itself clinically first in the bones of the head, then the chest, and finally in the long bones. Similarly rickets may be detected roentgenographically and histologically when it cannot be detected clinically. In our work the width of the metaphyses of the distal end of the radii and ulnæ seems to be the most sensitive indicator. The percentage of ash in the femurs varies much less on this ration. These variations, while interesting in themselves, are, however, of but minor significance to us at this time because our studies are comparative rather than absolute. In other words, we are concerned more with the relative values of the hays than we are with the fact as to whether the animal was absolutely normal or not.

*Experiments with Goats.*

In addition to the data secured with rats metabolism work was also carried out with milking goats and here we chose Hay 3; that is, the hay which had been exposed to the weather including dew and rain as well as sunlight. We chose this hay because of its doubtful value—on account of its extreme weathered condition—and because the farmer is frequently forced to use hay of this quality on account of the difficulty of curing clover in the field. We not only desired to test the calcifying power of this hay with a milking animal (see Tables III to IV, Period 1), but we also wanted to know whether such a hay could have its antirachitic potency augmented by direct exposure to artificial ultra-violet light to a sufficient degree to satisfy the requirements of a milking animal. In preparation for the irradiation the hay was cut rather coarsely ($\frac{1}{2}$ to 1 inch in length) in the first trial. It was irradiated *en masse* with ultra-violet light for 1 hour.

This hay as seen in Tables III to IV, Period 2, gave positive calcium balances in the 3rd week of feeding, indicating a decided activation of the hay as effected by its exposure to ultra-violet light. Apparently, however, the activity was not great enough to bring on a positive calcium balance earlier. In a second trial

the hay was finely ground and irradiated in a thin layer with the same source of ultra-violet light as before.

The second period of feeding shows that the irradiation of the more finely ground material improved its antirachitic properties. This comparison is seen in Period 2 in Tables III to V where the records of the results of the irradiated hay are shown. Though not too much emphasis is to be put upon the time of incidence of

TABLE III.

*Record of Calcium Balance and Milk Production of Goat I.*

| Date. | Dried feces. | CaO in feces. | CaO in urine. | CaO in milk. | Total CaO excreted. | Total CaO intake. | Balance per week. | Milk per week. |
|---|---|---|---|---|---|---|---|---|
| | | | Clover Hay 3. Period 1. | | | | | |
| | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *cc.* |
| Jan. 20–27 | 1435 | 25.40 | 0.04 | 20.30 | 45.74 | 39.78 | −5.96 | 7520 |
| " 27–Feb. 3 | 1460 | 30.81 | 0.08 | 17.87 | 48.76 | 39.20 | −9.56 | 6620 |
| Feb. 3–10 | 1325 | 29.42 | 0.07 | 14.90 | 44.39 | 39.20 | −5.19 | 5960 |
| " 10–17 | 1395 | 32.36 | 0.06 | 13.52 | 45.94 | 39.20 | −6.74 | 5410 |
| | | | Clover Hay 3. Irradiated with ultra-violet light for 1 hr. (hay coarse). Period 2. | | | | | |
| Feb. 17–24 | 1390 | 30.99 | 0.07 | 11.57 | 42.63 | 39.20 | −3.43 | 4820 |
| " 24–Mar. 3 | 1390 | 29.74 | 0.02 | 11.16 | 40.92 | 38.53 | −2.39 | 4465 |
| Mar. 3–10 | 1355 | 24.80 | 0.08 | 9.83 | 34.71 | 39.20 | +4.49 | 4275 |
| | | | Clover Hay 3. Animal irradiated 20 min. daily. Period 3. | | | | | |
| Mar. 10–17 | 1407 | 24.20 | 0.04 | 10.12 | 34.36 | 39.20 | +4.84 | 4050 |
| " 17–24 | 1365 | 23.75 | 0.07 | 9.24 | 33.06 | 39.20 | +6.14 | 3850 |
| " 24–31 | 1380 | 22.59 | 0.05 | 7.96 | 30.60 | 39.20 | +8.60 | 3620 |

the positive balance because the effect of irradiation may be cumulative, yet it is seen that irradiation of the finely ground material for 1 hour produced a positive balance in the 2nd week of feeding; while with the irradiated coarse hay it will be remembered that a positive balance did not ensue until the 3rd week of feeding.

In addition to the experiments involving the direct feeding of Hay 3 and feeding the same hay after irradiation with ultra-violet light, it was considered desirable to determine whether

TABLE IV.

*Record of Calcium Balance and Milk Production of Goat II.*

| Date. | Dried feces. | CaO in feces. | CaO in urine. | CaO in milk. | Total CaO excreted. | Total CaO intake. | Balance per week. | Milk per week. |
|---|---|---|---|---|---|---|---|---|
| Clover Hay 3. Period 1. | | | | | | | | |
| | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *cc.* |
| Jan. 20–27 | 1375 | 25.57 | 0.16 | 16.47 | 42.20 | 39.78 | −2.42 | 6335 |
| " 27–Feb. 3 | 1395 | 27.20 | 0.14 | 15.71 | 43.05 | 39.20 | −3.85 | 5820 |
| Feb. 3–10 | 1365 | 28.25 | 0.32 | 12.99 | 41.56 | 39.20 | −2.36 | 5370 |
| " 10–17 | 1370 | 29.04 | 0.21 | 12.91 | 42.16 | 39.20 | −2.96 | 5165 |
| Clover Hay 3. Irradiated with ultra-violet light for 1 hr. (hay coarse). Period 2. | | | | | | | | |
| Feb. 17–24 | 1385 | 29.08 | 0.38 | 11.57 | 41.03 | 39.20 | −1.83 | 5030 |
| " 24–Mar. 3 | 1410 | 29.32 | 0.29 | 11.23 | 40.84 | 39.20 | −1.64 | 4680 |
| Mar. 3–10 | 1375 | 26.02 | 0.22 | 9.32 | 35.56 | 39.20 | +3.14 | 4240 |
| Clover Hay 3. Animal irradiated 20 min. daily. Period 3. | | | | | | | | |
| Mar. 10–17 | 1380 | 26.41 | 0.20 | 9.39 | 36.00 | 39.20 | +3.20 | 4270 |
| " 17–24 | 1370 | 24.11 | 0.25 | 9.13 | 33.49 | 39.20 | +5.71 | 3970 |
| " 24–31 | 1330 | 23.00 | 0.18 | 7.74 | 30.92 | 39.20 | +8.38 | 3870 |

TABLE V.

*Record of Calcium Balance and Milk Production of Goat III.*

| Date. | Dried feces. | CaO in feces. | CaO in urine. | CaO in milk. | Total CaO excreted. | Total CaO intake. | Balance per week. | Milk per week. |
|---|---|---|---|---|---|---|---|---|
| Clover Hay 3. Period 1. | | | | | | | | |
| | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *cc.* |
| Apr. 22–29 | 1392 | 26.58 | 0.07 | 14.21 | 40.86 | 39.20 | −1.66 | 7530 |
| " 29–May 6 | 1470 | 27.49 | 0.08 | 12.78 | 40.35 | 39.20 | −1.15 | 6390 |
| May 6–13 | 1365 | 29.80 | 0.05 | 11.76 | 41.61 | 39.20 | −2.41 | 6190 |
| Clover Hay 3. Irradiated with ultra-violet light 1 hr. (hay ground fine). Period 2. | | | | | | | | |
| May 13–20 | 1380 | 30.63 | 0.07 | 11.94 | 42.64 | 39.20 | −3.24 | 5230 |
| " 20–27 | 1415 | 26.46 | 0.14 | 10.37 | 37.04 | 39.20 | +2.16 | 5460 |
| " 27–June 3 | 1275 | 23.20 | 0.13 | 10.54 | 33.87 | 39.20 | +5.33 | 5550 |
| June 3–10 | 1372 | 26.07 | 0.11 | 9.80 | 35.98 | 39.20 | +3.22 | 5160 |

positive calcium balances could be obtained by direct irradiation of the animal. Consequently during Period 3 the hay which was exposed to the weather was fed, but the goats themselves were directly irradiated with ultra-violet light for 20 minutes daily. These results are also shown in Tables III and IV, Period 3.

During all these experiments the milking goats were fed a grain mixture consisting of 59 parts of yellow corn, 25 of wheat bran, 15 of oil meal, and 1 of common salt. The entire ration as fed consisted of 2 parts of the grain mixture to 1 part of the clover hay, the hay constituting 33⅓ per cent of the ration. A study of the records secured with the goats (see Tables III to V) shows that this hay cured for 14 days in the weather possessed comparatively low calcifying power. Positive calcium balances were not established during its feeding.

*Chicken Experiments.*

To extend further our knowledge on the antirachitic value of the different hays and to explore experimentally as to other methods which might be used in this field of experimentation, we attempted to use the chick as the experimental animal. The chick being a rapidly growing animal has inordinately large requirements for the antirachitic factor, but unfortunately it is not so constituted that it can consume large amounts of a bulky food such as hay. This necessitated the use of extracts. In the absence of data on the quantitative extractability of the antirachitic factor, we provisionally assumed that ether is a suitable solvent as is suggested by the work of Shipley, Kinney, and McGollum (7). It is, however, not safe to conclude from their work nor from ours that all the antirachitic factor is extracted by the ether because in other experiments we have experienced difficulty in extracting the antirachitic vitamin quantitatively from a plant material even with such a solvent as alcohol. Quantitative extraction can only be concluded when the residue fed directly gives no evidence of activity. We believe, however, that our data with the chick have at least the value of helping us to decide on *modus operandi* in future experiments.

Our experiments were run during the months of March and April, 1925, in our dimly lighted attic. The birds used were 1 day old Barred Rocks. They were fed basally our rickets-pro-

TABLE VI.

*Record of Ash Content of Tibia of Chicks.*

| Chick No. | Weight of bird. | Age. | Ash of tibia. |
|---|---|---|---|
| | Control. Basal ration. | | |
| | *gm.* | *wks.* | *per cent* |
| 903 | 95 | 5 | 32.4 |
| 904 | 130 | 5 | 37.9 |
| 905 | 145 | 5 | 34.7 |
| 906 | 120 | 5 | 32.7 |
| 907 | 120 | 5 | 31.8 |
| 908 | 120 | 5 | 37.6 |
| 909 | 100 | 5 | 35.3 |
| 910 | 115 | 5 | 34.2 |
| 911 | 125 | 5 | 42.7 |
| Average | | | 35.5 |
| | Basal ration + ether extract of Hay 1 (attic-cured). | | |
| 932 | 160 | 5 | 33.4 |
| 933 | 145 | 5 | 34.0 |
| 934 | 110 | 5 | 37.9 |
| 935 | 130 | 5 | 34.4 |
| 936 | 115 | 5 | 37.7 |
| 937 | 115 | 5 | 39.1 |
| 938 | 110 | 5 | 35.1 |
| 939 | 140 | 5 | 32.1 |
| 940 | 155 | 5 | 35.0 |
| Average | | | 35.4 |
| | Basal ration + ether extract of Hay 2 (exposed to sunlight 14 days—kept dry). | | |
| 922 | 140 | 5 | 40.6 |
| 923 | 135 | 5 | 35.3 |
| 924 | 110 | 5 | 36.4 |
| 925 | 145 | 5 | 37.2 |
| 926 | 130 | 5 | 31.2 |
| 927 | 115 | 5 | 36.2 |
| 928 | 130 | 5 | 37.9 |
| 929 | 115 | 5 | 33.8 |
| 930 | 150 | 5 | 35.8 |
| 931 | 115 | 5 | 30.6 |
| Average | | | 35.5 |

TABLE VI—*Concluded.*

| Chick No. | Weight of bird. | Age. | Ash of tibia. |
|---|---|---|---|
| Basal ration + ether extract of Hay 3 (exposed to weather 14 days). | | | |
| | *gm.* | *wks.* | *per cent* |
| 912 | 150 | 5 | 34.4 |
| 913 | 155 | 5 | 37.7 |
| 914 | 130 | 5 | 37.8 |
| 915 | 115 | 5 | 31.4 |
| 916 | 135 | 5 | 33.2 |
| 917 | 110 | 5 | 34.7 |
| 918 | 140 | 5 | 34.7 |
| 919 | 135 | 5 | 34.1 |
| 920 | 110 | 5 | 30.0 |
| 921 | 105 | 5 | 36.7 |
| Average | | | 34.5 |

ducing ration of 97 parts of yellow corn, 2 of calcium carbonate, 1 of sodium chloride, and skimmed milk *ad libitum*, the latter being taken from cows which had been stabled on dry feed since January, 1925. In addition to this ration there was fed the ether extract of the hay in such amounts that to each gm. of the grain-salts mixture there was added the equivalent of 1 gm. of clover hay. This extract was prepared by extracting the hay from 4 to 6 hours in a large Soxhlet with ether. The extract was evaporated directly on the ration.

One group of birds of ten each was run with each one of the hays and in addition one group was run as a control. At 5 weeks of age when they all showed evidence of leg weakness, they were killed with ether and the tibiæ removed for ash analysis. The bones were dried at 50°C. for 24 hours; crushed with a pair of pliers; then extracted with hot 95 per cent alcohol for 36 hours; dried and ashed. The results are shown in Table VI. They indicate uniformly that the hay extracts did not improve the basal ration for the chick.

## DISCUSSION.

When considered directly, it is evident that the rat represents a most sensitive reactor to the antirachitic factor when this is added to our standard rickets-producing ration No. 2965.

Certainly our results with it have been far more analytical than those obtained with the goat and chick whose needs are apparently of such order that no difference in reaction to rather large additions was secured. It has been our experience in all our work that the chick and the lactating goat require rather large amounts of the antirachitic factor before any difference in their calcium metabolism results. Undoubtedly in the goat we have the complicating factor of the secretion of the antirachitic principle with the milk, but it is also very probable that there are distinct species variations in the requirement of animals for this constituent. It is, however, necessary to emphasize the fact that the amounts of antirachitic factor needed by an animal are not by any means absolute. They vary with the inorganic constituents of the diet. We (8) pointed this out some time ago in connection with our rat studies which showed that on low calcium rations the amount of cod liver oil necessary to furnish the antirachitic factor in sufficient amounts varied inversely with the calcium content of the diet. This was true on high phosphorus diets. On excessively high calcium diets we undoubtedly have the reverse situation. In the absence of facts bearing on these contingencies, it is rather beside the point to emphasize apparent species variations. We simply want to indicate that progress in this field can be facilitated most by experimental studies with the rat and then the further utilization of these results in planning experiments with other animals. It is most certainly not a debatable matter that we must know the requirement of man on an ordinary practical diet as well as that of our economically important animals.

We consider it a significant matter that our data have shown that hays vary tremendously in antirachitic content depending upon their exposure to sunlight. This immediately makes it intelligible why we should have obtained such variations in experimental results on calcium balances in our former trials. At that time we believed that exposure to the weather acted only deleteriously on certain essential factors elaborated by the plant and it appeared probable to us that the hay cured under caps possessed superior nutritive value because it had been protected from excessive exposure. It now, however, appears probable that the hay cured under caps was of superior nutritive value

because it had been more completely activated antirachitically by exposure to the sun. This raises the entire question of how hay should be cured so as to yield a product of maximum nutritive value. It appears necessary "to make hay when the sun shines," but it is to be remembered that excessive exposure of hay to sunlight in the field destroys vitamin A and then there are other factors to be considered. Here may be included the questions: What is the effect of curing hay under caps? What is the effect of heating in the mow and of aging? And furthermore, Why is it that green grass is not more active antirachitically? These questions need to be answered because of the prominent rôle which calcium plays in the animal body.

To a certain extent it appears possible to use artificial illumination with ultra-violet radiations to impart antirachitic properties to feeds. It remains, however, to be seen whether this is economically possible under present agricultural conditions. The direct irradiation of the animal promises to be a more satisfactory procedure and the time is probably not far distant when it will be common practice in animal husbandry to irradiate animals periodically which by circumstance cannot be directly exposed to sunlight.

It is needless to point out that the results of these studies have their application to human physiology both directly and indirectly.

## SUMMARY.

1. The antirachitic properties of hays are related to their exposure to sunlight. Clover hay made with exposure to sunlight showed considerable calcifying power, while the clover hay made in the dark was inactive.

2. Clover hay excessively weathered was reduced in antirachitic action as compared with hay less exposed to dew and rain.

3. The antirachitic action of the clover hay excessively weathered was readily increased by irradiating it with a quartz mercury vapor light. It is not clear whether this was due to an activation of constituents formerly inactive or to a reactivation.

4. Direct irradiation of a milking goat with ultra-violet light from a quartz mercury vapor lamp brought about a positive calcium balance even when the animal was receiving the weathered clover which by itself had no beneficial effect.

5. The antirachitic potency of the sun-cured hays was too feeble to act as a preventative of rickets in growing chicks even when the ether extracts were fed as equivalent to a high concentration of the hay in the ration. It is suggested that the antirachitic requirements of the chicken and the lactating goat are far greater than those of the rat as used in these experiments.

6. On account of the prominent rôle played by the element calcium in animal physiology, it appears urgently necessary that the factors operative in producing a hay of maximum antirachitic potency be thoroughly understood. This is especially necessary because calcium deficiency in feeds is a common occurrence and with that there is a proportionate increased demand of the animal for the antirachitic factor. There remains no question of the advisability of "making hay when the sun shines."

7. These studies are of value directly as well as indirectly in their relation to physiological processes in the human.

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EXPLANATION OF PLATE 1.

On the left, internal view of costochondral junctions from Rat 2445, Lot 3189. This rat received a rickets-producing ration carrying 5 parts of Clover Hay 1 cured in the dark.

On the right, internal view of costochrondral junctions from Rat 2449, Lot 3190. This rat received the same ration but the clover had previously been exposed to ultra-violet radiations.

(Steenbock, Hart, Elvehjem, and Kletzien: Calcium assimilation. VI.)

# FAT-SOLUBLE VITAMIN.

## XXVI. THE ANTIRACHITIC PROPERTY OF MILK AND ITS INCREASE BY DIRECT IRRADIATION AND BY IRRADIATION OF THE ANIMAL.*

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Some time ago we (1) made the statement that cow's milk, well known to be an antirachitic agent of questionable value, can have its antirachitic potency materially increased by direct exposure to the radiations of a quartz mercury vapor lamp. Hess (2) has made a similar statement and Cowell (3) has used irradiated milk successfully in stimulating calcium deposition in children. It appeared desirable that the data of our experiments which brought out this fact should be published in detail because they indicate again to what extremes the animal organism is dependent upon ultra-violet radiations for the endowment of its food with the requisite antirachitic properties; and because they also suggest the possibility of a practical utilization of the principle in the preparation of infant foods. This latter is not, however, without its practical difficulties as will be pointed out later, and therefore when considered from the standpoint of increasing the antirachitic properties of milk alone, we believe that direct irradiation of the animal has much to offer. We shall discuss this later.

### EXPERIMENTAL.

Our first experiments were carried out in the month of February, 1925, with cow's milk obtained from stall-fed cows. These cows received a mixture of grains, silage, and alfalfa as their regular

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ration and were allowed to exercise in a paddock exposed to the sun for a few hours daily when the weather was not too cold or disagreeable.

As a test for antirachitic properties the milk from these cows was fed in quantities of 0.5 to 12 cc. daily to rachitic rats. For irradiation 50 cc. of milk were poured into a galvanized iron pan measuring 12 by 24 inches, which was then tipped from side to side so as to distribute the milk as evenly as possible. It was exposed to ultra-violet light generated by a quartz mercury vapor burner of the Cooper-Hewitt BY type at a distance of 2 feet for 30 minutes. The burner was run at a burner voltage of 40 to 60 and 4 amperes.

The rats used for the tests were animals of our own breeding started at approximately 60 gm. in weight on our rickets-producing ration No. 2965. This, as we have already published (4), consists of 76 parts of yellow corn, 20 of wheat gluten, 3 of calcium carbonate, and 1 of sodium chloride. It always produces rickets of uniform severity in our rats in a few weeks. After 29 days on this ration, by which time moderate rickets had developed in all our rats, their diets were supplemented for 10 days by the addition of milk measured out individually into small glass dishes. The rats were then killed and the distal ends of their radii and ulnæ split with a scalpel and stained with silver nitrate. In most cases the bones were not stained immediately after dissection because it was found that fixing in 10 per cent formalin, not only had no harmful effects, but gave much clearer preparations. The results of these experiments tabulated in Table I show that it took 12 cc. of non-irradiated milk to produce a response in our animals, but only 1 cc. of the irradiated.

It was a question in our minds whether or not we had irradiated our milk sufficiently long to secure maximum results. Purposely we had used as thin a film of milk as we could obtain by pouring it into a large pan, but we were apprehensive lest a prolonged exposure might result in an inactivation such as we had experienced with oils (4). Arbitrarily we selected 30 minutes as the period of time because that had given us optimum results in previous trials with other foods (4).

Table II gives the results of feeding milk exposed to our quartz mercury vapor light for periods varying from 0 to 120 minutes.

This time 50 cc. of milk were exposed in a smaller pan (a circular pie tin $7\frac{3}{4}$ inches in diameter) because the pans used before were

TABLE I.

*The Increase of the Antirachitic Properties of Cow's Milk by Irradiation.*

| Lot No. | Rat No. | Milk added daily. | Weight when changed. | Final weight. | Average daily consumption. | Calcium deposition. |
|---|---|---|---|---|---|---|
| | | | Non-irradiated. | | | |
| | | *cc.* | *gm.* | *gm.* | *gm.* | |
| 3143 | 2217 | 0.5 | 98 | 92 | 6 | − |
| | 2218 | 1.0 | 104 | 108 | 5–6 | − |
| | 2219 | 2.0 | 88 | 101 | 4 | − |
| | 2220 | 4.0 | 105 | 118 | 6 | − |
| | 2221 | 8.0 | 115 | 137 | 6 | ? |
| | 2222 | 12.0 | 100 | 102 | 3–4 | + |
| 3144 | 2223 | 0.5 | 88 | 90 | 5–6 | − |
| | 2224 | 1.0 | 95 | 102 | 6–7 | − |
| | 2225 | 2.0 | 112 | 123 | 8 | − |
| | 2226 | 4.0 | 96 | 104 | 6 | − |
| | 2227 | 8.0 | 96 | 109 | 5 | + |
| | 2228 | 12.0 | 107 | 122 | 5 | + |
| | | | Irradiated. | | | |
| 3145 | 2229 | 0.5 | 97 | 109 | 8 | ? |
| | 2230 | 1.0 | 85 | 94 | 5 | + |
| | 2231 | 2.0 | 94 | 100 | 7 | + |
| | 2232 | 4.0 | 95 | 92 | 6 | + |
| | 2233 | 8.0 | 89 | 88 | 4–5 | + |
| | 2234 | 12.0 | 96 | 105 | 4 | + |
| 3146 | 2235 | 0.5 | 85 | 85 | 6 | − |
| | 2236 | 1.0 | 94 | 98 | 6 | + |
| | 2237 | 2.0 | 98 | 102 | 5–6 | + |
| | 2238 | 4.0 | 88 | 85 | 5 | + |
| | 2239 | 8.0 | 80 | 75 | 5 | + |
| | 2240 | 12.0 | 100 | 109 | 5–6 | + |

too large to allow simultaneous exposure of a number of samples. The rats were given 1 cc. of the milks daily for 10 days after they had been on our Ration 2965 for 3 weeks. The table shows that 30 minutes gave us optimum results in activation.

Our next experiments were carried out with goat's milk because we wanted to determine not only the extent to which its antirachitic properties could be increased by exposing it to ultraviolet light, but also the effect of direct irradiation of the animal. For such an experiment a goat is obviously a more convenient animal than a cow.

Goat I, a young goat which had been confined in a dimly illuminated basement all winter, was used for the first trials beginning in the month of April, 1925. She weighed 50 pounds and was producing an average of 213 cc. of milk daily. Previous to the experiment and during the experiment she received all she would consume of a ration of 2 parts of a grain mixture and 1 part of chopped alfalfa hay. The grain mixture was composed of 35 parts of corn-meal, 30 parts of bran, 30 parts of ground oats, and 5 parts of linseed oil meal.

The goat was exposed to the quartz mercury vapor arc for 30 minutes daily when irradiation was started. Exposure was made at a distance varying from 12 to 30 inches from the lamp depending upon whether the animal was standing up or lying down. As her coat was exceedingly heavy, the long hair was clipped from her back before irradiation. This did not, however, expose her skin because her coat was almost felt-like in thickness.

Milk was fed to rachitic rats before irradiation and after, beginning respectively 4, 9, and 17 days after irradiation was started. In addition samples of milk irradiated directly were fed as controls to determine the degree of activation possible. In all cases the rachitic rats were prepared as before, being fed our rickets-producing ration No. 2965 for 21 to 28 days before milk was added to the diet. Milk was fed for a period of 10 days. It was measured out individually for each rat.

The results of these trials are presented in Table III in abbreviated form to conserve space. The table is self-explanatory. It shows that while it took 12 cc. of control milk to bring about incipient calcium deposition, after irradiation of the milk it took only 0.5 cc. and after irradiation of the animal 2 cc. Evidently irradiation of the animal was not so effective as direct irradiation of the milk itself, but nevertheless it is noteworthy that the increase in potency was sixfold. This appears the more remarkable when it is taken into consideration that the milk was taken

beginning 4 days after irradiation was started and ending after 14 days irradiation, by which time the animal had received a total of only 7 hours of exposure.

By longer treatment with ultra-violet radiations no further increase in activity occurred, but it was a noticeable fact that the animal consumed its ration with greater avidity after such treatment. It became more active and increased in weight in 4 weeks

TABLE II.

*Time of Exposure Necessary to Activate Cow's Milk Completely. 1 Cc. Fed to Each Rat Daily.*

| Lot No. | Rat No. | Basal ration. | Days on basal ration. | Time of exposure of milk. | Days milk was fed. | Weight when changed. | Final weight. | Average daily consumption. | Calcium deposition. |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *min.* | | *gm.* | *gm.* | *gm.* | |
| 3374 | 3377 | 2965 | 21 | 0 | 10 | 100 | 118 | 7–8 | − |
| | 3378 | 2965 | 21 | 5 | 10 | 85 | 101 | 7–8 | − |
| | 3379 | 2965 | 21 | 10 | 10 | 88 | 108 | 7–8 | − |
| | 3380 | 2965 | 21 | 30 | 10 | 91 | 98 | 6 | ++ |
| | 3381 | 2965 | 21 | 60 | 10 | 81 | 89 | 6–7 | ++ |
| | 3382 | 2965 | 21 | 120 | 10 | 92 | 103 | 7 | + |
| 3375 | 3383 | 2965 | 21 | 0 | 10 | 73 | 86 | 6 | − |
| | 3384 | 2965 | 21 | 5 | 10 | 87 | 78 | 6–7 | − |
| | 3385 | 2965 | 21 | 10 | 10 | 80 | 91 | 6 | − |
| | 3386 | 2965 | 21 | 30 | 10 | 79 | 87 | 6 | ++ |
| | 3387 | 2965 | 21 | 60 | 10 | 70 | 78 | 5–6 | ++ |
| | 3388 | 2965 | 21 | 120 | 10 | 70 | 80 | 6 | ++ |

− no calcium deposition.
+ narrow line of calcium.
++ average line of calcium.

from 50 pounds to 56 pounds. Evidently the resultant changes in the physiology of the animal were more deep seated than mere increase in the antirachitic potency of its milk. We have already pointed out how animal tissues (5) become endowed with antirachitic properties by irradiation and how an animal can also metabolize calcium salts with greater efficiency after such treatment (6).

The rapidity with which the milk is affected in antirachitic properties as a result of irradiation of the animal is shown in Table IV. This presents results obtained with Goat II.

Goat II weighed 93 pounds. She had dropped her kid about 4 weeks before she was used for the experiment and had been kept for months on a ration of grain and alfalfa in the dimly lighted basement of our barn. She produced from 700 to 1000 cc. of milk daily.

TABLE III.

*Goat I. Calcium Deposition in Rachitic Rats after Feeding Milk from an Irradiated Goat.*

| Amount of milk fed daily. | Milk not irradiated. | | Milk irradiated 30 min. | | Milk from goat irradiated 30 min. daily. Feeding of milk begun after irradiation of: | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | 4 days. | 9 days. | | 17 days. | |
| cc. | | | | | | | | | |
| 0.2 | | | — | — | | | | | |
| 0.5 | | | +? | ++ | | | | | |
| 1.0 | — | — | ++ | ++ | — | — | — | — | — |
| 2.0 | — | — | +++ | ++ | + | +? | ++ | +? | — |
| 4.0 | — | — | ‡ | ‡ | ++ | + | + | + | ++ |
| 6.0 | — | — | | | ++ | +++ | ‡ | + | +++ |
| 8.0 | −? | −? | ‡ | ‡ | +++ | +++ | ‡ | ‡ | ‡ |
| 12.0 | + | ‡ | | | ‡ | +++ | ‡ | ‡ | ‡ |

— no calcium deposition.
\+ narrow line of calcium.
++ average line of calcium.
+++ wide line of calcium.
‡ union with diaphysis.

The experimental procedure with this animal was about the same as before, the important exceptions being that the animal was irradiated 1 hour daily instead of ½ hour and that samples of milk for the 1, 2, and 4 day periods were sterilized daily for ½ hour at 15 pounds steam pressure and then kept in the refrigerator during the 10 day feeding interval. For the 8th day period the milk was, however, again taken daily and fed directly. The rachitic rats used for the test were prepared in the same way as before on Ration 2965 on which they were kept from 21

to 22 days before the milk additions were made. For comparative purposes one series of rats was given milk drawn before irradiation and another the same after it had been irradiated as in the previous experiment.

The results are presented in Table IV. They suggest that the milk was somewhat improved in antirachitic properties by the 2nd day of irradiation and by the 7th day maximum activity had apparently been imparted. The 8 to 18 day samples gave no better results. With this animal the effect of irradiation was apparently not so pronounced as with Goat I because it took 4 cc.

TABLE IV.

*Goat II. Calcium Deposition in Rachitic Rats after Feeding Milk from an Irradiated Goat.*

| Amount of milk fed daily. | Milk not irradiated. | Milk irradiated 30 min. | Milk from goat irradiated 1 hr. daily. Feeding of milk begun after irradiation of: | | | |
|---|---|---|---|---|---|---|
| | | | 1 day. | 2 days. | 4 days. | 8 days. |
| cc. | | | | | | |
| 0.2 | | − | | | | |
| 0.5 | | + | | | | |
| 1.0 | − | ++ | − | − | − | − |
| 2.0 | − | +++ | − | − | − | − |
| 4.0 | − | ++++ | − | +? | + | + |
| 6.0 | − | ++++ | − | + | + | ± |
| 8.0 | − | | − | ± | +++ | ± |
| 12.0 | ± | | ± | | | |

rather than 2 cc. of milk to produce an antirachitic response. Whether this is due to variation in responsiveness of the two animals, we are not in position to say. The animals were treated exactly alike except that Goat II was irradiated twice as long.

## DISCUSSION.

The data presented in this paper supplement our previously published data which showed that exposure of the animal to ultra-violet radiations effected an economy in its metabolism of calcium salts. These data make us realize that the offspring of irradiated animals may benefit as well and that such benefit may be secured indirectly through the medium of the milk. In 1924

Luce (7) studied the antirachitic properties of milk from the standpoint of its relation to sunlight and feed and came to the conclusion that the feed was the more important factor. We have come to realize that this may or may not be the case—depending entirely upon whether or not the feeds have been exposed to ultra-violet light. It does, however, appear to be true that sunlight acting upon the animal itself is not such an extremely active agent as one might be led to assume.

With the broadening of our conceptions of the rôle of calcium in the body and the realization that, at least in comparison to the animal's needs, calcium usually occurs in foods and feeds in quantities bordering on a deficiency, the part that the antirachitic factor plays becomes more and more impressive. We (8) have already emphasized the fact that to obtain a hay of first quality, it should be cured exposed to sunlight. In the light of data presented in this paper, it now appears justifiable to emphasize the fact that the producer of so called high grade milk has undoubtedly gone to extremes in protecting his cows from sunlight. This he has done not entirely by intent because his concern primarily has been to keep his herd away from the heat, flies, and dust. To effect this he has stabled his animals sometimes the year around. It is true he has seen the necessity of having his barns well provided with windows but unfortunately sunlight passing through glass is no longer antirachitic. The question immediately arises if some of the difficulties experienced in high producing herds, such as failure to breed, non-infectious abortion, and physical breakdown, may not have as a contributing cause, if not often their origin, a low calcium ration coupled with low antirachitic content. Outside of this it appears advisable that cows should be exposed liberally to direct sunlight in order that they may be able to produce a first class product for human consumption as well as for the production of good calves. In lieu of exposure to sunlight and for the sake of cleanliness and convenience, if not efficiency, the time is probably not far distant when every producer of high grade milk will find it necessary to irradiate his cows artificially.

The lessons to be drawn by the hygienist, the pediatrican, and the general practitioner of medicine from these studies are obvious.

SUMMARY.

By exposure to the radiations of a quartz mercury vapor lamp, the antirachitic properties of cow's milk were increased eight or more times. Under the same conditions a sample of goat's milk increased in activity about twenty-four times. This increase in activity can also be induced rather promptly, though to a lesser degree, by direct irradiation of the animal. Attention is called to the beneficial results to be expected by the dairyman. For man in general the indirect benefits are obvious.

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# ANTIRICKETIC SUBSTANCES.

## II. THE ACTION OF *n*-BUTYL NITRITE ON ACTIVATED CHOLESTEROL AND THE ANTIRICKETIC VITAMIN.

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Following somewhat separate lines of inquiry, three groups of investigators have recently associated the antiricketic[1] vitamin with a derivative of cholesterol produced by ultra-violet radiations. Hess, Weinstock, and Helman (1), Rosenheim and Webster (2), and Steenbock and Black (3) have in turn produced the antiricketic derivative by exposing cholesterol to the rays of the mercury vapor quartz lamp. Although the suggested hypothesis that this derivative is identical with the natural vitamin is not without foundation, further data are required for its confirmation or confutation.

If it can be demonstrated that a substance, X, is destroyed by a substance, Y, and a substance, Z, under similar conditions is not destroyed by Y, then it follows that X and Z are not identical; but if both X and Z are destroyed by Y, they may or may not be identical, and the reaction merely suggests their *similarity*. I have previously shown (4) that the antiricketic vitamin in cod liver oil is destroyed by nitrous fumes; in the present communication this reaction is applied to activated cholesterol.

[1] Since the English noun "rickets" affords a better connotation than does the etymologically unrelated term "rachitis" it would seem to the author that "antiricketic" or "antirickets" is better usage than "antirachitic." This detail of orthography appears to have been generally overlooked.

*Methods.*

The facilities of a laboratory especially designed for nutritional studies made it possible to overcome certain of the difficulties in attaining uniform experimental conditions. In a future paper a more extensive discussion of technique will be given, but the present experiments were performed as follows: Young rats were fed the McCollum rickets-producing diet, No. 3143. This diet appears to be sensitively constituted, but when prepared and employed as described it produced satisfactory rickets. All the

*Diet 3143.*

| | |
|---|---|
| Whole wheat flour (Pillsbury's "100 per cent") | 33 |
| Yellow maize (seed quality, finely ground) | 33 |
| Wheat gluten (Pure Gluten Food Company) | 15 |
| Bacteriological gelatin ("Difco," powdered form) | 15 |
| Calcium carbonate (Baker "analyzed," heavy powder) | 3 |
| Sodium chloride (Eimer and Amend "tested purity," powder) | 1 |

rats developing rickets, and those receiving curative preparations, were kept in metal cages in a sanitary room without windows. The only light employed was from ruby glass bulbs, the illumination being that of a photographic dark room. The temperature was maintained at about 30°C. Under these conditions the rats developed severe rickets in 18 days.

Curative preparations were incorporated at a definite per cent by weight by trituration with Diet 3143. In all cases the period of administration was 7 days, and five rats weighing at least 50 gm. were used for each test. Experience has shown that to secure statistical reliability with this technique it is necessary to discard the records of all rats which (*a*) lose weight during the 7 day treatment, and which (*b*) eat less than 2 gm. of food in any 1 day or less than 3 gm. per day for the 7 day period.

The protocols of the rats are combined in Table I. Experimental findings were obtained by the Shipley "line test" procedure, in which a longitudinal section through the proximal end of the tibia was illuminated in silver nitrate until the calcified structures blackened. The principal criteria of healing are the development of the "line" at the zone of provisional calcification and the reappearance of bony trabeculæ in the metaphysial osteoid. For convenience in tabulation the degree of healing is expressed on a

scale of four plus signs, which the reader should realize bear no numerical relation to each other.

## EXPERIMENTAL.

In my original investigation of the nitrous fumes reaction (4) I employed the oxides of nitrogen generated by the interaction of sodium nitrite and acetic acid. In the present work I found that the reaction can be made more precise by using *n*-butyl nitrite, which is freely miscible with oils and can be accurately measured. The cod liver oil used in this experiment was found to effect advanced healing when administered at 4 per cent, this dose being, however, more than sixteen times the minimum required to initiate healing in 7 days.

*Action of n-Butyl Nitrite on Cod Liver Oil.*—8 gm. of cod liver oil were mixed with 1 ml. of *n*-butyl nitrite, and kept for 1 hour in a small test-tube at room temperature (29°). A calculated quantity of the mixture was then triturated with Diet 3143 so as to give a product containing 4 per cent of oil. This preparation was exposed to air for 2 hours to dissipate the odor. When fed to rats it induced advanced healing, which shows that the antiricketic vitamin in cod liver oil is not rapidly destroyed by *n*-butyl nitrite at room temperature.

A second tube of cod liver oil and *n*-butyl nitrite was prepared the same as the first, except that this was allowed to stand for 95 hours. In this time the oil became dark reddish brown, and when administered at 4 per cent it induced no healing. It is evident that *n*-butyl nitrite slowly destroys the antiricketic vitamin in cod liver oil at room temperature.

A third tube of oil and nitrite was prepared the same as the first, except that this tube was immediately plugged with cotton and immersed in boiling water for 1 hour. Bubbles of gas developed, and the oil quickly darkened. The tube was promptly cooled, and the product made up to 4 per cent as before. The tests with rats indicated that the antiricketic vitamin in cod liver oil is destroyed by heating the oil with *n*-butyl nitrite for 1 hour. A control tube of oil heated without nitrite gave advanced healing at 4 per cent. This observation is the basis for the subsequent examination of activated cholesterol.

TABLE I.

For convenience in tabulation, the quality of healing observed by the line test is here expressed on a scale of four degrees. One plus sign (+) indicates a just perceptible healing; two plus signs (++), a distinct healing; three plus signs (+++), an advanced healing; and four plus signs (++++), a practically complete healing. The reader should realize that these values bear no numerical relation to each other.

| Rat No. | Sex. | Preparation administered. | Grade of test. | Weight. | Average daily consumption. |
|---|---|---|---|---|---|
| | | | | *gm.* | *gm.* |
| 1013 | M. | Diet 3143 control. | – | 64–64 | 3.9 |
| 1014 | " | " " " | – | 52–57 | 5.0 |
| 1015 | F. | " " " | – | 59–61 | 3.9 |
| 1016 | M. | " " " | – | 58–61 | 4.4 |
| 1069 | F. | " " " | – | 51–59 | 5.9 |
| 1045 | F. | Cod liver oil 4 per cent. | ++++ | 53–54 | 3.9 |
| 1046 | " | " " " " | ++ | 52–54 | 4.0 |
| 1073 | " | " " " " | ++++ | 50–50 | 3.6 |
| 1074 | M. | " " " " | +++ | 52–53 | 3.7 |
| 1075 | F. | " " " " | ++++ | 52–53 | 4.9 |
| 1029 | M. | Cod liver oil ¼ per cent. | +++ | 60–61 | 3.7 |
| 1030 | " | " " " " | +++ | 53–61 | 4.7 |
| 1031 | F. | " " " " | +++ | 56–58 | 3.9 |
| 1032 | " | " " " " | +++ | 56–61 | 4.4 |
| 1033 | M. | " " " " | +++ | 51–53 | 3.3 |
| 1098 | M. | Cod liver oil 4 per cent with *n*-butyl nitrite 1 hr. cold. | ++++ | 64–65 | 5.3 |
| 1114 | " | " " " " | +++ | 68–68 | 5.6 |
| 1140 | " | " " " " | ++++ | 66–67 | 4.6 |
| 1141 | F. | " " " " | +++ | 52–53 | 4.4 |
| 1142 | M. | " " " " | ++++ | 55–58 | 5.6 |
| 1057 | F. | Cod liver oil 4 per cent with *n*-butyl nitrite 95 hr. cold. | – | 56–56 | 3.3 |
| 1058 | M. | " " " " | – | 52–53 | 3.4 |
| 1105 | " | " " " " | – | 58–58 | 5.0 |
| 1157 | F. | " " " " | – | 70–75 | 5.9 |
| 1170 | M. | " " " " | – | 53–59 | 4.4 |

TABLE I—*Concluded.*

| Rat No. | Sex. | Preparation administered. | Grade of test. | Weight. | Average daily consumption. |
|---|---|---|---|---|---|
| | | | | *gm.* | *gm.* |
| 1119 | M. | Cod liver oil 4 per cent with *n*-butyl nitrite 1 hr. hot. | − | 59–60 | 5.3 |
| 1120 | " | " " " | − | 58–58 | 6.0 |
| 1160 | " | " " " | − | 70–73 | 5.1 |
| 1161 | F. | " " " | − | 63–65 | 4.4 |
| 1162 | M. | " " " | − | 56–61 | 4.7 |
| 1052 | F. | Cod liver oil 4 per cent heated 1 hr. without *n*-butyl nitrite. | +++ | 55–55 | 3.4 |
| 1053 | M. | " " " | +++ | 54–54 | 3.7 |
| 1056 | F. | " " " | ++++ | 55–56 | 3.7 |
| 1076 | " | " " " | +++ | 52–52 | 6.0 |
| 1077 | " | " " " | ++++ | 57–59 | 3.9 |
| 1048 | M. | Seal oil 4 per cent. | − | 55–58 | 3.4 |
| 1049 | F. | " " " | − | 55–60 | 4.6 |
| 1050 | " | " " " | − | 56–58 | 4.0 |
| 1051 | M. | " " " | − | 64–65 | 4.4 |
| 1068 | " | " " " | −(?) | 60–64 | 4.9 |
| 1017 | F. | Seal oil 4 per cent plus cholesterol $\frac{1}{10}$ per cent. | − | 53–54 | 3.4 |
| 1018 | M. | " " " | − | 57–58 | 3.9 |
| 1019 | " | " " " | − | 60–64 | 3.9 |
| 1020 | " | " " " | − | 58–60 | 4.4 |
| 1110 | " | " " " | −(?) | 55–57 | 6.1 |
| 1024 | M. | Seal oil 4 per cent plus irradiated cholesterol $\frac{1}{10}$ per cent. | ++ | 64–68 | 3.9 |
| 1025 | " | " " " | + | 53–55 | 4.3 |
| 1026 | " | " " " | ++ | 54–54 | 3.4 |
| 1027 | " | " " " | ++ | 59–60 | 4.1 |
| 1028 | " | " " " | + | 61–65 | 4.1 |
| 1034 | M. | Cod liver oil 4 per cent plus irradiated cholesterol $\frac{1}{10}$ per cent with *n*-butyl nitrite 1 hr. hot. | − | 64–64 | 4.0 |
| 1035 | " | " " " | − | 63–66 | 4.1 |
| 1036 | " | " " " | − | 61–61 | 3.7 |
| 1038 | " | " " " | − | 67–70 | 4.3 |
| 1039 | " | " " " | − | 62–66 | 4.0 |

*Preparation of Cholesterol.*—The cholesterol was originally obtained from cod liver oil by Wilkins and Bills in the course of some unpublished investigations on the antiricketic vitamin. The crude product was purified by repeated crystallization from hot alcohol and treatment with decolorizing charcoal. The final product consisted of large, colorless, thin laminæ. The melting point of the anhydrous material, determined by the conventional capillary tube method, was 147° corrected. One must be especially careful in determining this constant for cholesterol, because the pulverized crystals include so much air that sintering may obscure the true melting point.

*Activation of Cholesterol.*—The cholesterol was activated in the dry, powdered state by exposing a very shallow layer for 15 minutes to the radiations from a Cooper Hewitt horizontal "Uvi-arc" operated at 150 volts. The burner was 36 cm. from the cholesterol. After irradiation, the cholesterol powder was thoroughly stirred to insure uniformity throughout the mass.

*Properties of Seal Oil.*—To determine the biological effect of the cholesterol before and after irradiation, and to secure results comparable with those obtained with 4 per cent of cod liver oil, it was decided to dissolve the cholesterol in a similar quantity of an inactive oil. Mineral or vegetable oil might suffice, but a marine oil similar to cod liver oil seemed preferable.

It is noteworthy that seal oil, which chemically and physically resembles cod liver oil, and is sometimes used to adulterate the latter, is not appreciably antiricketic. Through the kindness of Messrs. Job Brothers and Company, Ltd., of Saint John's, Newfoundland, a supply of pure "pale" seal oil was obtained. This oil was said to have been rendered the 1st week in April, 1925, by briefly steaming the fresh pelt fat of the young harp seal (*Phoca grænlandica*). After settling, the oil was bleached in tanks by exposure to sunlight for a few days. As received in this laboratory, it was almost colorless and odorless. Administered to rats at 4 per cent, its antiricketic value was nil.

*Action of n-Butyl Nitrite on Activated Cholesterol.*—Purified cholesterol was dissolved in seal oil and incorporated in Diet 3143 in such proportions that the modified diet contained 4 per cent of seal oil and 0.1 per cent of cholesterol. This preparation induced no definite healing, which shows that the purified cholesterol was

inactive. A second modified diet was prepared the same as the first, except that the irradiated cholesterol was employed. This gave a moderate healing in all five rats, which shows that the irradiated cholesterol was antiricketic. Contemporaneously a third modified diet was prepared as follows: 200 mg. of the same irradiated cholesterol were dissolved in 8 gm. of cod liver oil in a small test-tube. 1 ml. of *n*-butyl nitrite was added, and the tube plugged with cotton and immersed in boiling water for 1 hour. The cooled product was triturated with Diet 3143 in such proportions that the modified diet contained 4 per cent of cod liver oil and 0.1 per cent of irradiated cholesterol. The line tests were negative, which shows that the antiricketic potency of irradiated cholesterol, like that of cod liver oil, is destroyed by *n*-butyl nitrite.

SUMMARY.

1. The antiricketic vitamin in cod liver oil was destroyed by *n*-butyl nitrite, slowly at room temperature, and rapidly on heating.

2. Some seal oil was found to be inactive, but irradiated cholesterol dissolved in the seal oil was moderately antiricketic. When the irradiated cholesterol was dissolved in cod liver oil and heated with *n*-butyl nitrite the antiricketic activity was destroyed.

3. The above reactions support, but do not prove, the hypothesis that the antiricketic vitamin is identical with the antiricketic derivative of cholesterol produced by irradiation.

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# STUDIES ON CHOLESTEROL.

## I. SYNTHESIS OF CHOLESTEROL IN THE ANIMAL BODY.*

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### INTRODUCTION.

The question of the synthesis of cholesterol by the animal body has long been open to discussion. Whether or not this ever present constituent of protoplasm must be included in the diet has never been definitely determined. According to Gardner (1) and his coworkers the phytosterols of the plant foods are transformed to cholesterol in the body, furnishing a portion at least of this substance. This, however, cannot be said to constitute a synthesis. Can the animal body build up cholesterol from substances other than sterols? To throw some light upon this question has been the purpose of this investigation.

The work of others in this field has resulted in somewhat conflicting views. Ellis and Gardner (2) in 1909 from a comparison of the cholesterol content of eggs and of newly hatched chicks concluded that there was no synthesis. Beumer (3) also found no evidence of a synthesis in hen's eggs during development of the chick. On the other hand, Thannhauser and Schaber (4) reported the opposite results from similar experiments. Gardner and Lander (5) found no evidence of cholesterol synthesis in chickens fed on an extracted diet. At a considerably later time, however, Gardner and Fox (6) from experiments on human subjects fed over 10 day periods concluded that "there must be some organ in the body capable of synthesising cholesterol." In 1913 Dezani (7) reported that white mice given cholesterol-free food showed distinct undernourishment compared with mice fed on an unextracted diet. In later experiments (8) with white rats he succeeded in keeping animals for 10 weeks on an extracted diet of corn-

* A report of this work was presented before the American Chemical Society, September, 1924, Proceedings of the Division of Biological Chemistry (*Science*, 1924, lx, 530).

meal and casein. These animals gave evidence of a cholesterol synthesis in that their cholesterol content was roughly double that of controls killed at the beginning of the experiment. Gamble and Blackfan (9) compared the cholesterol intake with the excretion in infants over 3 day periods. They found in all experiments a much larger excretion than intake and interpreted the result as indicating a synthesis of cholesterol. This evidence does not seem very conclusive, however, because of the fact that the experiments were continued over such very short periods of time. Recently, Beumer and Lehmann (10) have reported experiments upon newly born dogs which were fed for 4 weeks on a diet low in cholesterol and found an increase in that time amounting to twenty times the amount of cholesterol furnished in the diet. They infer, therefore, that cholesterol was synthesized from substances not found in the ether extract of the food.

This brief review of the literature apparently leaves the question of cholesterol synthesis undecided, but with the bulk of evidence on the affirmative side. It does not appear, however, that anyone has yet used a diet free from cholesterol,[1] and at the same time adequate for maintenance and growth. Nor, so far as we are aware, has anyone carried the experimental animals over more than one generation. These two points seem to us to be important in arriving at a definite conclusion.

## EXPERIMENTAL.

In carrying out our experiments the white rat was chosen as the experimental animal. Young rats at the time of weaning were placed on a diet free from cholesterol and all other sterols which respond to the Liebermann-Burchard acetic anhydride-sulfuric acid color test. These rats were allowed to reproduce and their young were continued on the cholesterol-free diet. At the age of about 5 months the original cholesterol-free rats were killed and the cholsterol content of the brains, blood, and livers was determined. For comparison purposes like determinations were made on rats of the same age which had been kept on exactly the same diet with a definite amount of pure cholesterol added. Rats which had been kept on a normal breeding ration were also treated in the same way. The offspring from both the cholesterol-free rats and the controls were continued until about 5 months of age

[1] Since this work was reported similar results have been obtained by Channon working with rats on a cholesterol-free diet. Channon, H. J., *Biochem. J.*, 1925, xix, 424.

when they were killed for purposes of analysis. In several cases the whole carcass of the rat, less the skin, head, and viscera, was minced in a meat grinder and analyzed for cholesterol content. Finally, a number of young, 1 day old, from both the cholesterol-free and control animals were killed and analyzed. In this way it seemed possible to show quite definitely whether or not the organism of the rat could build up its own cholesterol.

Before this could be done, however, it was necessary to devise a diet which should be free from cholesterol and yet contain all the other necessary factors in proper amount. There seemed to be one main difficulty in preparing such a diet. This was to find a source of the fat-soluble vitamin A which would be free from cholesterol. Since both cholesterol and vitamin A are found in the non-saponifiable fraction of any fat containing the vitamin it proved very difficult to free any of these fats from cholesterol without at the same time removing the vitamin A. Advantage was finally taken of the findings of Steenbock and Boutwell (11) in their studies of the efficiency of various solvents in extracting vitamin A from green plant tissues. They found that ether was a rather poor extracting solvent when used in the cold on such substances as alfalfa, carrots, and corn. Alfalfa, for example, was found to contain considerable amounts of vitamin A even after repeated extractions with cold ether. It seemed likely, therefore, that by prolonged extraction of alfalfa with cold ether the sterols could be removed leaving sufficient vitamin A for normal growth.

To test this point a quantity of alfalfa was cut while in bloom, brought to the laboratory, and dried at room temperature. The leaves and small branches were stripped from the stalks and ground to a fine powder. This powder was extracted with cold ether in an apparatus modeled somewhat upon the Soxhlet extraction apparatus, but of such size as to contain about 800 gm. of alfalfa powder. The extraction time was 72 hours. The extracted alfalfa was washed three times with ether and dried in the air. The dry extracted alfalfa was then tested for its content of vitamin A. When present in the diet to the extent of 10 per cent, it was found to induce normal growth in rats suffering from lack of vitamin A.

A diet was then made up of the following composition: Alcohol-extracted casein, 18 per cent; corn starch, 58 per cent; extracted

alfalfa, 15 per cent; salt mixture (McCollum No. 185), 4 per cent; yeast extract (Vegex), 5 per cent. A portion of this diet was dried and extracted with ether in a Soxhlet extraction apparatus for 24 hours. The extract gave no qualitative test for cholesterol by either the colorimetric or the digitonin method. At a later time samples of this diet prepared in the same way but from different lots of material were hydrolyzed with both acid and alkali and extracted with boiling alcohol. These extracts were also tested by both the colorimetric and digitonin methods with negative results.

Eight rats were selected from good growing stock and placed on this diet at the time of weaning. These rats were about 1 month old and weighed from 60 to 65 gm. each. Two males and two females were placed in each cage. The cages were provided with screen bottoms to prevent the eating of feces. Four other rats were placed at the same time on the same diet with 0.05 per cent cholesterol added. At a later date other control rats were started on diets containing 0.025 per cent and 0.1 per cent of added cholesterol. One cage of rats was also kept on the ration diet. These rats were all weighed twice a week for about 4 months. At about 5 months of age they were killed after taking a sample of blood from each, and the cholesterol content of the liver, brains, and blood was determined.

In making the cholesterol determination on the blood Bloor's (12) method was followed. The blood obtained from two rats was mixed and about 3 or 4 cc. were extracted with 100 cc. of an alcohol-ether mixture as described in the method. In the case of the other tissues, the livers and brains were removed and weighed as quickly as possible and the livers of two rats were ground together in plaster of Paris. This was then extracted in a Soxhlet apparatus with ether for 24 hours. The ether was evaporated off and the extract dried at about 50–60°C. It was then taken up in alcohol-ether mixture (3 : 1) and made up to a definite volume. The cholesterol determinations were then made in the same way as upon a blood extract. The brain extracts were prepared in the same way. Table I shows the results of the analyses on the rats kept upon the cholesterol-free diet, those upon the same diet with the known amounts of cholesterol added, and those upon the ration diet.

TABLE I.

*Cholesterol Found in Tissues.*

| Rat No. | Diet. | Age when killed. | Blood. | | Brains. | | Livers. | |
|---|---|---|---|---|---|---|---|---|
| | | | Cholesterol per 100 cc. | Average. | Cholesterol per 100 gm. | Average. | Cholesterol per 100 gm. | Average. |
| | | mos. | gm. | gm. | gm. | gm. | gm. | gm. |
| 162, 164 | Cholesterol-free. | 5 | 0.076 | | 1.35 | | 0.148 | |
| First generation. 163, 168 | " | 5 | 0.077 | | 1.36 | | | |
| First generation. 156, 165 | " | 9 | 0.101 | | 1.57 | | 0.184 | |
| First generation. 155, 157 | " | 11 | 0.088 | | 1.40 | | 0.147 | |
| Second generation. 180, 181 | " | 5 | 0.076 | | 1.54 | | 0.203 | |
| Second generation. 191, 192 | " | 5 | 0.081 | 0.083 | 1.41 | 1.44 | 0.170 | 0.169 |
| 195, 196 | Cholesterol-free plus 0.025 per cent cholesterol. | 5 | 0.100 | | 1.32 | | 0.149 | |
| 193, 194 | " " " | 8 | 0.080 | | 1.58 | | 0.176 | |
| 231, 235 | " " " | 5 | 0.075 | 0.085 | 1.44 | 1.44 | 0.192 | 0.172 |
| 158, 159 | Cholesterol-free plus 0.05 per cent cholesterol. | 5 | 0.079 | | 1.29 | | | |
| 167, 170 | " " " | 5 | 0.083 | 0.081 | 1.04 | 1.165 | 0.131 | 0.131 |
| 199, 200 | Cholesterol-free plus 0.1 per cent cholesterol. | 5 | 0.083 | | 1.31 | | 0.096 | |
| 197, 198 | " " " | 5 | 0.092 | 0.087 | 1.33 | 1.32 | 0.098 | 0.097 |

TABLE I—*Concluded.*

| Rat No. | Diet. | Age when killed. | Blood. | | Brains. | | Livers. | |
|---|---|---|---|---|---|---|---|---|
| | | | Cholesterol per 100 cc. | Average. | Cholesterol per 100 gm. | Average. | Cholesterol per 100 gm. | Average. |
| | | *mos.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* |
| 189<br>185 | Ration 0.03 per cent cholesterol by analysis. | 5½ | 0.074 | | 1.27 | | 0.125 | |
| 149R<br>150R | " " " | 5 | 0.078 | 0.076 | 1.43 | | | |
| 83<br>84 | " " " | 9 | | | 1.48 | 1.39 | 0.165 | 0.145 |

In addition to these analyses, the cholesterol content of a number of young rats (1 day old) born on these various diets was determined. The young rats were weighed and after being killed were ground in plaster of Paris in the manner described for the brain and liver analyses. Table II shows the amount of cholesterol found in the 1 day old rats from the several sources.

The average weight of the day old rats was about 5 gm. The total cholesterol content of the entire body of a day old rat born on the cholesterol-free diet was approximately 5.5 mg. In the brains alone of rats born on the same diet and kept on this diet until 5 months old cholesterol was found to the extent of 21 to 24 mg. In other words, the brain of a rat born and raised to 5 months of age on a cholesterol-free diet was found to contain more than four times as much cholesterol as the entire body of the rat at birth.

Determinations were also made of the cholesterol content of the entire body exclusive of head, skin, and viscera of two rats 9 months old, kept from the time of weaning on a cholesterol-free diet. Like analyses were made on four other rats, two of which had been kept on the ration diet and two on the cholesterol-free diet plus 0.025 per cent cholesterol added. The results of these analyses are shown in Table III.

As previously stated, all analyses were made by the colorimetric

method. The suggestion was made that some other substance was giving this color and not cholesterol. That such was not the case was shown by preparing from one of the brain extracts the characteristic cholesterol crystals apparently free from impurities. These crystals responded readily to the usual cholesterol tests.

TABLE II.

*Cholesterol Found in 1 Day Old Offspring.*

| Diet of parent rats. | No. of young analyzed. | Cholesterol found (average). | |
|---|---|---|---|
| | | *per cent* | *gm.* |
| Cholesterol-free | 3 | 0.110 | 0.0051 |
| " + 0.025 per cent cholesterol | 3 | 0.128 | 0.0053 |
| " + 0.1 " " " | 3 | 0.162 | 0.0087 |
| Ration diet 0.03 per cent cholesterol by analysis. | 8 | 0.0894 | 0.0051 |

TABLE III.

| Rat No. | Diet. | Weight of bodies less head, skin, and viscera. | Cholesterol found. | Total cholesterol in bodies. |
|---|---|---|---|---|
| | | *gm.* | *per cent* | *gm.* |
| 156<br>165 | Cholesterol-free. | 300 | 0.094 | 0.282 |
| 186<br>187 | Ration. | 300 | 0.106 | 0.320 |
| 193<br>194 | Cholesterol-free + 0.025 per cent cholesterol. | 255 | 0.124 | 0.315 |

DISCUSSION.

From the experimental evidence presented, it seems reasonable to conclude that the rat is able to synthesize cholesterol. The amount of cholesterol found in the tissues of the cholesterol-free rats examined does not seem to vary greatly from the normal. There is some variation in the results of the separate analyses but similar variations are found in the results obtained from the control animals. On averaging the figures for each group of rats it is found that the differences are slight, inconsistent, and of no significance. In any event, there seems to be no question but that the

cholesterol content of the adult rat born and raised on a cholesterol-free diet is many times as great as at birth, and the only explanation available at present is that the cholesterol was built up by the animal organism from substances other than sterols in the diet.

SUMMARY.

1. The preparation of a diet free from cholesterol is described.
2. Experimental evidence is submitted to show that the organism of the white rat is able to synthesize cholesterol.

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# THE DESTRUCTIVE ACTION OF ACIDS, ALKALIES, AND ENZYMES ON INSULIN.*

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While a considerable amount of work has been reported on the destruction of insulin by acids, alkalies, and proteolytic enzymes, little if any data are available on the quantitative study of the reactions either with respect to the rate of destruction or the end-products of the reaction. Earlier work in this laboratory[1] with moderate sized samples failed to furnish us with reliable data.

When larger amounts of commercial insulin became available, an investigation of the quantitative changes produced by the action of acid, alkali, and trypsin on insulin was undertaken. Insulin, Lot 92523, which was used for the entire data reported here, contained 9.40 mg. of organic solids per cc., 1.48 mg. of nitrogen per cc., of which 0.294 mg. was non-protein nitrogen. Each cc. contained 200 units or 21 units per mg. of solids. The entire physiological activity was contained in that fraction which was precipitated by 50 per cent saturation with $ZnSO_4$ in an acid medium. While more recent work has resulted in the preparation of insulin of a much higher degree of purity, the data secured in this experiment are of such a character that we did not feel it advisable to sacrifice more material for this phase of the investigation.

The amount of nitrogen contained in the fractions of insulin precipitated by definite concentrations of $ZnSO_4$ and by tungstic acid, in the case of the non-protein nitrogen, was determined.

* A summary of a phase of the investigation of the chemical nature of insulin carried out in this laboratory during the summer and autumn of 1923. All of the material used was manufactured by Eli Lilly and Company under the trade name of Iletin. Iletin (Insulin-Lilly), *J. Am. Med. Assn.*, 1923, lxxx, 1851. Part of this investigation was reported at the American Association for the Advancement of Science meeting in Cincinnati, 1923.

[1] Shonle, H. A., and Waldo, J. H., *J. Biol. Chem.*, 1923–24, lviii, 731.

Then, after the several hydrolyses, the amount of nitrogen and the number of units remaining in these same fractions were determined, and the rate and character of the hydrolysis were compared with the loss in activity.

The insulin solution was diluted ten to fifteen times its volume with water and enough $H_2SO_4$ added to make the solution 0.25 per cent acid by weight. The acidified solution was then placed

TABLE I.

| $ZnSO_4$ saturation. | N precipitated per cc. of original solution. | | | |
|---|---|---|---|---|
| | 0 hr. | 1 hr. | 2 hrs. | 3 hrs. |
| *per cent* | *mg.* | *mg.* | *mg.* | *mg.* |
| 20 | 0.300 | 0.167 | 0.167 | 0.104 |
| 30 | 0.660 | 0.254 | 0.230 | 0.201 |
| 50 | 0.850 | 0.283* | 0.375 | 0.222 |
| 100 | 0.870 | 0.468 | | 0.230 |
| Activity in units..... | 200 | | 60 | 40 |

* Apparently the low value is due to some analytical error.

TABLE II.

| $ZnSO_4$ saturation. | N precipitated per cc. of original solution. | | | | |
|---|---|---|---|---|---|
| | 0 hr. | 0.5 hr. | 1 hr. | 3 hrs. | 6 hrs. |
| *per cent* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* |
| 30 | 0.660 | 0.229 | 0.166 | 0.141 | 0.100 |
| 50 | 0.850 | 0.625 | 0.476 | 0.267 | 0.167 |
| 100 | 0.870 | 0.806 | 0.750 | 0.333 | 0.246 |
| Total protein N*..... | 1.186 | 1.187 | 1.145 | 1.078 | 0.959 |
| Activity in units..... | 200 | 105 | 105 | 45 | 30 |

* The total nitrogen minus the non-protein nitrogen represents total protein N in all tables.

under a reflux condenser, brought rapidly to the boiling point, and refluxed gently. Samples were removed at the indicated time and both the physiological activity and the amount of nitrogenous material precipitated by varying concentrations of $ZnSO_4$ and by tungstic acid were determined. Since the actual weight of the $H_2SO_4$ added was four times that of the insulin, there was little change in the H ion concentration during hydrolysis. The data are contained in Tables I and II and in Chart 1.

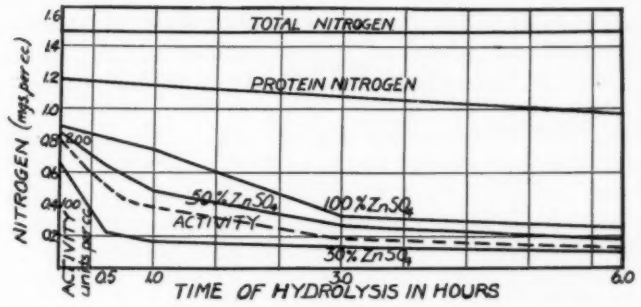


CHART 1. Effect of 0.25 per cent $H_2SO_4$ on insulin at 100°C.

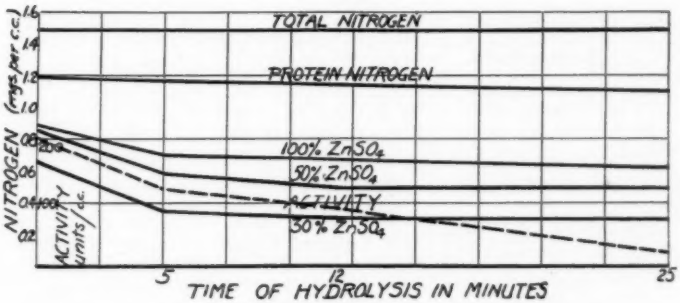


CHART 2. Effect of alkali on insulin. NaOH pH = 8.6 at 100°C.

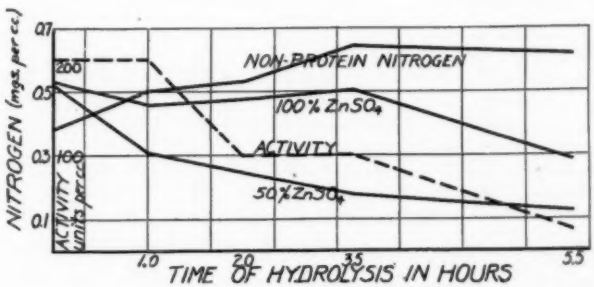


CHART 3. Effect of trypsin on insulin at 37°C.

In the second series of experiments the insulin was diluted ten times and the pH adjusted to 8.6 to 9.0 by the careful addition

TABLE III.

| $ZnSO_4$ saturation. | N precipitated per cc. of original solution. | | | |
|---|---|---|---|---|
| | 0 min. | 5 min. | 12 min. | 22 min. |
| *per cent* | *mg.* | *mg.* | *mg.* | *mg.* |
| 30 | 0.660 | 0.128 | 0.131 | 0.125 |
| 50 | 0.850 | 0.452 | 0.374 | 0.305 |
| 100 | 0.870 | 0.625 | 0.546 | 0.514 |
| Total protein N | 1.186 | 1.113 | 1.105 | 1.059 |
| Activity in units | 200 | 80 | 25 | 10 |

TABLE IV.

| $ZnSO_4$ saturation. | N precipitated per cc. of original solution. | | | |
|---|---|---|---|---|
| | 0 min. | 5 min. | 12 min. | 25 min. |
| *per cent* | *mg.* | *mg.* | *mg.* | *mg.* |
| 30 | 0.660 | 0.357 | 0.306 | 0.307 |
| 50 | 0.850 | 0.572 | 0.487 | 0.487 |
| 100 | 0.870 | 0.694 | | 0.632 |
| Total protein N | 1.186 | 1.170 | 1.146 | 1.113 |
| Activity in units | 200 | 120 | 90–100 | 20–30 |

TABLE V.

| $ZnSO_4$ saturation. | N precipitated per cc. of original solution. | | | |
|---|---|---|---|---|
| | 0 min. | 5 min. | 12 min. | 25 min. |
| *per cent* | *mg.* | *mg.* | *mg.* | *mg.* |
| 30 | 0.660 | 0.300 | 0.347 | 0.340 |
| 50 | 0.850 | 0.720 | 0.625 | 0.625 |
| 100 | 0.870 | 0.870 | 0.700 | 0.780 |
| Total protein N | 1.186 | 1.166 | 1.207 | 1.197 |
| Activity in units | 200 | 100 | 50 | 30 |

of a 1 per cent solution of sodium hydroxide. The solution was brought to boiling rapidly under a reflux. Samples were removed at the indicated time and assayed. No attempt was made to

maintain a constant pH due to the rapidity of the reaction.[2] The data are contained in Tables III, IV, and V and in Chart 2.

In the third series of experiments, the insulin,[3] was diluted and mixed with a suspension of Armour's trypsin equal in weight to 10 per cent of the insulin solids. The pH was then reduced to 8 and a slight amount of an insoluble flocculent precipitate filtered off. The filtrate was then sampled and placed in an incubator at at 37°C. without any preservative. Previous work had demonstrated that no loss of activity resulted from bacterial action during a brief incubation.[4] Samples were removed at the indicated time and assayed. Table VI and Chart 3 give the data secured.

TABLE VI.

| $ZnSO_4$ saturation. | N precipitated per cc. of original solution during digestion. | | | | |
|---|---|---|---|---|---|
| | 0 hr. | 1 hr. | 2 hrs. | 3.5 hrs. | 5.5 hrs. |
| *per cent* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* |
| 30 | 0.197 | | | | |
| 50 | 0.524 | 0.302 | 0.246 | 0.177 | 0.113 |
| 100 | 0.530 | 0.463 | 0.476 | 0.509 | 0.297 |
| Non-protein N | 0.384 | 0.500 | 0.515 | 0.624 | 0.609 |
| Activity in units | 200 | 200 | 100 | 100 | 20 |

[2] Witzemann, E. J., and Livshis, L., *J. Biol. Chem.*, 1923–24, lviii, 463, find that ammonium hydroxide inactivates insulin in such a way that subsequent acid treatment can usually recover the activity. We were able in some instances to reduce the activity of a sample to one-sixth by the addition of a suitable amount of ammonium hydroxide, and after acidification to recover one-half of the original activity. In other instances no recovery was possible.

[3] Insulin 92523 contained no preservative.

[4] The destruction of the insulin was not due to the alkalinity of the solution, since control experiments show no loss of activity during 6 hours incubation at 37°. Epstein, A. A., and Rosenthal, N., *Am. J. Physiol.*, 1924, lxx, 225, conclude that trypsin inactivates insulin almost instantaneously, but that by proper acidification, the insulin can be liberated from the trypsin. They state that prolonged contact of trypsin with insulin does not destroy the insulin, even 42 hours incubation at 37.5°C., and that the action of trypsin on insulin is not of a proteolytic character.

Although convulsions were secured, the doses given by Epstein and Rosenthal at the expiration of the periods of digestion were equivalent to 20 units per rabbit, and the conclusion that no destruction occurred cannot be upheld since under the conditions of their experiment a 90 or 95 per cent

EXPERIMENTAL.

The method of Zunz[5] was followed in separating the insulin into fractions for nitrogen analysis. According to this method, the solution containing the protein material is made 0.75 per cent acid by the addition of sulfuric acid, and enough $ZnSO_4$ is then added to secure the desired degree of saturation. Complete saturation precipitates all proteoses, while half saturation precipitates only the secondary proteoses. The precipitate secured by 30 per cent saturation was used to follow the rate of disintegration going on in the primary proteose fraction.

At the determined time, as shown in the tables, suitably sized samples were removed for chemical analysis and mixed with sufficient amounts of $H_2SO_4$ and $ZnSO_4$ to secure the desired degree of saturation. After 1 hour the precipitation was found to be complete (due to the low concentration of protein material present) and the precipitate was then filtered off and washed with the same concentration of acid $ZnSO_4$ solution. The precipitate was dissolved in 0.2 per cent HCl and the N determined on an aliquot portion by the micro-Kjeldahl method. The non-protein nitrogen was determined in another sample after the protein had been precipitated by sodium tungstate and sulfuric acid.[6] The nitrogen determinations were run either in duplicate or triplicate.

Samples for physiological standardization were likewise removed at the same time and placed at once in ice water and the pH brought to 3±. They were then injected into rabbits. From eight to twenty rabbits were used to determine the activity

destruction might have occurred and still they would have secured convulsions.

We recently repeated their work and found that insulin could be inactivated by the addition of trypsin and that acidifications caused a recovery, but that on digestion a progressive loss of activity occurred accompanied by proteolysis. The small amount of activity which persisted after a prolonged digestion could not be increased by acidification. Scott, D. A., *J. Biol. Chem.*, 1925, lxiii, 641, has recently shown that acid treatment after the digestion of trypsin and insulin does not recover the potency of the insulin. The initial reversible adsorption phenomena of insulin and trypsin become irreversible after digestion.

[5] Zunz, E., *Z. physiol. Chem.*, 1899, xxvii, 219; *Ann. Soc. sc. Belgique*, 1900, ix, pt. 2–3, as described in Effront, J., Biochemical catalysts in life and industry; translated by Prescott, S. C., New York, 1917, 221, 234.

[6] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

of each sample. One-third the amount of insulin which caused a convulsion in a 2 kilo rabbit was taken as the unit. With such a limited number of rabbits the true activity of the samples may vary as much as 15 to 20 per cent of the figure recorded.

## DISCUSSION.

In every instance it is observed that the loss of physiological activity was gradual and occurred simultaneously with the gradual hydrolysis of the protein material or as we have called it the primary proteose fraction.[7] In no instance was it possible to recover any of the activity although many attempts were made; *i.e.*, the change was irreversible. Although the rate of the reaction varied in the several experiments, a similarity is observed between the rapid breaking down of the 50 per cent $ZnSO_4$ fraction and the loss of activity.[8] It may be argued that, since we know that there are some inactive proteoses present, the hydrolytic cleavage may in no way be connected with the destruction of the activity. However, we find a gradual loss of activity due to the action of acid, alkali, or trypsin, whose one action in common is hydrolytic cleavage. Just as the rate and type of the hydrolytic cleavage of proteins by acid, alkali, and enzymes differ so do we find the rate of the destructive action differing.

[7] The insulin of commerce does not necessarily exist as such in the pancreas, since the process of extraction is such that the acid would undoubtedly have converted the natural proteins into such derivatives as acid proteins and the higher proteoses. While purified insulin has some of the characteristics of both the proteins and primary proteoses, it seems to fall within the latter classification. Purified insulin will not coagulate with heat, it will slowly dialyze through a parchment membrane, the precipitate which occurs on the addition of nitric or trichloroacetic acid disappears in warming to reappear again on cooling. While some investigators have reported that they have secured negative tests for protein in physiologically active solutions, this would appear to be due to the fact that the sensitivity of the physiological test is greater than the sensitivity of the protein test. 0.01 mg. of pure insulin can be readily detected physiologically.

[8] Insulin warmed with NaOH gives the usual test for reduced sulfur. However if the insulin has first been partially hydrolyzed with acid, the test is only a fourth as intense showing that the hydrolytic cleavage of the alkali and acid is quite different. Abel, J. J., and Geiling, E. M. K., *J. Pharmacol. and Exp. Therap.*, 1925, xxv, 423, report that insulin readily splits off reduced sulfur on warming with mild alkali in a manner which is not characteristic of proteins.

CONCLUSION.

Insulin may be of a true protein structure possessing specific physiological activity or it may consist of a chemical complex attached to a protein structure. However when the protein is broken down into slightly smaller units, by either acid, alkali, or enzyme, the physiological functioning is lost.

# THE QUANTITATIVE ESTIMATION OF TYROSINE AND HISTIDINE IN PROTEIN.

## A METHOD FOR ESTIMATING TYRAMINE IN PROTEIN-CONTAINING MIXTURES.

By MILTON T. HANKE.

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(Received for publication, September 12, 1925.)

### INTRODUCTION.

In 1922[1] Dr. Koessler and I published a colorimetric method for the quantitative estimation and separation of phenols, including tyrosine and tyramine. The method is based upon the color that is produced when phenols are allowed to react in alkaline ($Na_2CO_3$) solution with diazotized sulfanilic acid. The phenols tried, other than tyrosine and tyramine, give colors varying from yellow to red and the intensity of these colors is directly proportional to the amount of phenol introduced. This is not the case with tyrosine and tyramine.[2] An evanescent pink color appears which rapidly fades and becomes yellow. If, at this time, the liquid is treated with a solution of sodium hydroxide and then with a small amount of hydroxylamine hydrochloride, a brilliant violet color develops which reaches its maximum intensity within a few seconds and remains unchanged for 15 or more minutes. The amount of color so produced is directly proportional to the concentration of tyrosine or tyramine present. A search for interfering substances revealed the fact that tyrosine and tyramine are the only common phenols that give this reaction and that beside these two phenols, the reaction is obtained only with cer-

[1] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1922, l, 235, 271.

[2] See, however, Fürth, O., *Biochem. Z.*, 1924, cxlvi, 259. Using larger concentrations of tyrosine, the color production seems to be fairly proportional to the concentration of tyrosine.

tain volatile or easily decomposable enolic compounds such as acetone, acetoacetic acid or ester, and acetaldehyde.

The method is not applicable to the estimation of tyrosine or tyramine in protein and protein-containing mixtures because when other amino acids are present in sufficient quantity, they give a yellow color with the test reagent which masks the color produced by tyrosine or tyramine.

This reaction, which is so characteristic for tyrosine and tyramine, would serve as an ideal method of estimation in mixtures if the compounds could be at least partially freed from impurities before subjecting them to the colorimetric procedure.

Phenols have long been known to give insoluble compounds when they are heated, in aqueous solution, with mercuric acetate, the products being mercurized phenols with the mercury attached to a carbon atom of the benzene ring. A search of the literature revealed the fact that what one might presume to be such a compound, had been prepared[3] to which the formula $C_9H_9O_3NHg$ had been assigned. This compound was said to be insoluble in water and was used, therapeutically, after it had been dissolved in dilute alkali.

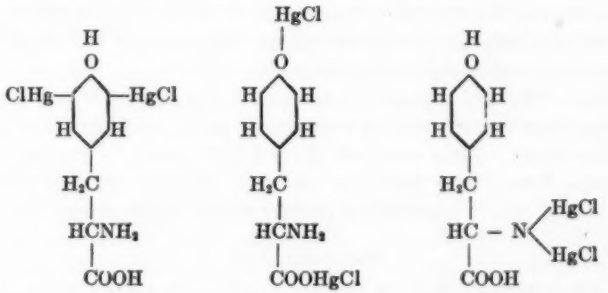
Attempts were made, therefore, to ascertain under what conditions an insoluble tyrosine mercury compound could be prepared and this has been accomplished. The tyrosine-containing mixture must first be freed from histidine, because histidine is precipitated, to some extent, by mercury under the conditions to be described. This can be most accurately accomplished by precipitating with silver sulfate and barium hydroxide. The histidine-free filtrate is freed from silver and barium and evaporated to dryness. The residue is suspended in 75 cc. of water, treated with 1 cc. of glacial acetic acid and 3.5 gm. of mercuric acetate, and boiled for 10 minutes under a reflux condenser or heated for 1 hour on the steam bath. If a quantity of *pure* tyrosine less than 100 mg. is present, a clear solution is obtained that remains clear when cooled. With protein hydrolysates a slight precipitate is always obtained which contains some, but not all, of the tyrosine. The cooled liquid, or mixture, is treated with 7.5 gm. of sodium chloride which precipitates the tyrosine as a fairly heavy amorphous white solid. The mixture is separated by centrifugalization and the insoluble resi-

[3] British patent No. 11,302 and U. S. patent Nos. 1,167,622 and 1,180,694.

due washed once with a small amount of a 10 per cent solution of sodium chloride. The clear supernatant liquids are discarded. They contain not more than 0.1 mg. of tyrosine. The mercury compound is dissolved in hot 20 per cent HCl, the solution diluted with water and freed from mercury by saturation with hydrogen sulfide. The mixture is filtered. The clear and usually colorless filtrate is freed from HCl by evaporation on the water bath. The residue is nearly pure tyrosine chloride. The amount of tyrosine present can be most conveniently estimated by the colorimetric process of Hanke and Koessler.[1]

The process thus briefly described fulfills all of the requirements for an exact determination. The tyrosine is first removed from everything that can possibly interfere with its colorimetric estimation and it is then estimated by an accurate colorimetric process.

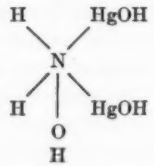
Before proceeding to a detailed description of the method and the results obtained with this method, something should be said about the tyrosine mercury compound. When properly isolated it has the molecular formula $C_9H_9Hg_2Cl_2O_3N$ for which three structural formulæ can be written; namely,




I. II. III.

The compound is formed rapidly only when the reaction liquid is heated. When washed exhaustively with water it loses a part or all of its chlorine. It gives a clear solution when boiled for a few minutes with $N \cdot H_2SO_4$, which remains clear when the liquid is cooled. Hydrogen sulfide precipitates the mercury quantitatively from this liquid. When the solid is suspended in water and the mixture saturated with hydrogen sulfide, the mercury is removed quantitatively.

These facts prove conclusively that the mercury atoms cannot be attached to carbon, which eliminates formula I. Formula II looks improbable. The fact that the compound is formed readily only when the reaction mixture is heated does not suggest salt formation. Such a compound would not be expected to exist in an acid solution because of the depressing action of the H ion on the reactivity of the phenolic hydrogen ion. The addition of NaCl, which converts the mercury into $NaHgCl_3$, reduces the concentration of the mercury ion almost to nil, which makes salt formation almost impossible. Formula III represents the configuration of the compound that is precipitated under the conditions of the experiment. It is a derivative of Millon's base.




It might be called *p*-hydroxyphenyl, $\alpha$-dichloromercuramino propionic acid or tyrosino-dimercuric chloride; but I shall refer to it subsequently as tyrosino-mercuric chloride and to its derivatives as tyrosino-mercury compounds.

Since the $\alpha$ amino group is common to all amino acids, one would expect that entirely similar compounds are formed with the other amino acids. They certainly do not precipitate to any marked extent, hence they may be soluble. Leucine—0.1 gm.—when treated as above precipitates to only a very slight extent.

## EXPERIMENTAL.

### *I. The Precipitation of Tyrosine and Tyramine Dimercuric Chloride.*

### *A. How Long Must Tyrosine Be Heated with Mercuric Acetate to Obtain a Quantitative Yield of Tyrosine Mercuric Chloride?*

Seven reaction mixtures were prepared containing:

0.05 gm. tyrosine.
75.00 cc. water.
1.00 " glacial acetic acid.
3.5 gm. mercuric acetate.

Five of the flasks were attached to reflux condensers and the contents boiled for 5 minutes, 30 minutes, 1 hour, 2 hours, and 3 hours respectively. One flask was placed on the steam bath and heated for 1 hour. One flask was not heated. The treatment in this one case is separately described.

The liquids that had been boiled for 2 and 3 hours, respectively, were slightly colored and contained a small amount of metallic mercury. The others were clear and nearly colorless.

The reaction liquids were cooled and treated with 7.5 gm. of sodium chloride which produced an immediate, flocculent but heavy, white precipitate. The mixtures were placed in the ice chest overnight, transferred to centrifuge tubes with 20 cc. of a 10 per cent solution of sodium chloride, and centrifuged. The solid separates readily. The clear supernatant liquid was removed by decantation and the solid washed once with 10 cc. of a 10 per cent solution of sodium chloride. The washed solid was transferred back to the boiling flask with 25 cc. of 20 per cent hydrochloric acid and heated on the water bath. A clear solution was obtained after heating for 30 minutes. Water—50 cc.—was then added. The liquid was saturated with hydrogen sulfide, the mixture heated on the water bath for 30 minutes, and filtered through a small folded filter. The filtrate and washings were collected in a glass dish and evaporated until all of the liquid had been removed. The pale brown residue was dissolved in water, diluted to 500 cc., and its tyrosine content estimated colorimetrically by the method of Hanke and Koessler.[1]

*Exactly 0.05 gm. of tyrosine was recovered in every case which is 100 per cent of the amount originally introduced.*

*The Unheated Reaction Liquid.*—In this case the tyrosine was dissolved in the water and acetic acid by boiling. The solution remained clear when it was cooled. It was then treated with 3.5 gm. of mercuric acetate and allowed to stand at room temperature for 30 minutes. The subsequent treatment with sodium chloride gave an opalescent solution but no precipitate. I feel justified in concluding that the reaction does not occur, to any appreciable extent, in 30 minutes at room temperature. Heat seems to be essential for the reaction; but the time of heating is, within certain limits, a matter of minor significance. Protracted boiling seems to produce a slight decomposition which is evidenced by the fact

that a small amount of metallic mercury separates out. In all of our subsequent experiments, therefore, the reaction liquid has either been boiled for 10 minutes or heated on the water bath for 1 hour.

*B. How Small an Amount of Tyrosine or Tyramine Mercuric Chloride Can Be Quantitatively Precipitated?*

Seven flasks were prepared as in A excepting that they contained, respectively, 10, 5, 2, and 1 mg. of tyrosine, and 10, 2, and 1 mg. of tyramine chloride. The liquids were boiled for 10 minutes and subjected to chemical treatment as given in A. The results are given in Table I.

TABLE I.

| | Amount introduced. | Amount recovered. |
|---|---|---|
| | *mg.* | *mg.* |
| Tyrosine. | 10 | 10 |
| | 5 | 5 |
| | 2 | 1.9 |
| | 1 | 0.812 |
| Tyramine. | 10 | 10 |
| | 2 | 1.88 |
| | 1 | 0.812 |

The loss of tyrosine or tyramine by this method is 0.1 to 0.2 mg. under the conditions of the experiment. This probably represents the limit of solubility of the compound. That perfect recoveries were obtained with the 5 and 10 mg. samples is not surprising because the limit of accuracy of the colorimetric methods is about 1.5 to 2 per cent. Since, in the average tyrosine determination, quantities considerably in excess of 5 mg. are determined, this solubility correction can be ignored entirely.

*C. Is Tyrosine Mercuric Chloride Soluble in a Solution That Contains Amino Acids? Are Insoluble Mercury Derivatives Obtained with Other Amino Acids? Is Any Tyrosine Precipitated before the Sodium Chloride Is Added?*

A mixture was prepared containing 0.1 gm. of each of the following amino acids: glycine, alanine, α-amino-*n*-valerianic acid,

valine, leucine, iso-leucine, aspartic acid, glutamic acid, phenylalanine, and tyrosine. The mixture was treated with 75 cc. of water and 1 cc. of glacial acetic acid and boiled under a reflux condenser until a clear solution had been obtained. Mercuric acetate—3.5 gm.—was then added and the boiling continued for 10 minutes. A precipitate formed during the first 30 seconds which increased in amount as the boiling continued; it was never large. The mixture was cooled, filtered through a very small filter, and the precipitate washed with 25 cc. of water.

*The Precipitate.*—This was suspended, paper and all, in 25 cc. of 20 per cent HCl and heated for 1 hour on the water bath. It was then diluted, saturated with hydrogen sulfide, etc., as described in A. The crystalline solid finally obtained was dissolved in water and diluted to 500 cc. It contained (colorimetric determination) 44.48 mg. of tyrosine.

*The Filtrate.*—This was treated with 10 gm. of sodium chloride, etc., as described in A. The solid finally obtained was dissolved in water and diluted to 1000 cc. It contained 56.2 mg. of tyrosine.

The total recovery of tyrosine was, therefore, 100.68 mg. which is 100.68 per cent of the amount originally introduced. A somewhat similar experiment was carried out on hydrolyzed gelatin with and without the addition of tyrosine. Of the added tyrosine, 99 per cent was recovered over and above that present in the gelatin.

*Conclusions.—Tyrosine can be quantitatively precipitated from a mixture of amino acids as tyrosine mercury derivative. Other amino acids are precipitated to only a very small extent,* possibly to some extent as components of the tyrosine mercury complex and to some extent as individual mercury compounds. *The amount of the other amino acids that are precipitated is too small to interfere in any way with the colorimetric estimation of tyrosine.*

### *D. To What Extent Do Chlorides and Sulfates Interfere with the Formation of Tyrosine Mercuric Chloride?*

The process to be described directly for estimating histidine and tyrosine in protein involves an adjustment to remove barium with sulfuric acid and silver with hydrochloric acid. The work is facilitated if the liquid can be so adjusted that it contains a slight excess of sulfate and chloride ions. It was necessary, therefore,

to prove that these two ions do not interfere with the *formation* of the mercury compound. Four solutions were prepared as in A containing 0.05 gm. of tyrosine. These were then treated with 0.45 and 0.9 gm. of sodium chloride and 0.71 and 1.42 gm. of sodium sulfate, respectively. The liquids were boiled for 10 minutes and otherwise treated and analyzed as in A.

*100 per cent of the tyrosine originally introduced was recovered in every case. Sodium chloride and sodium sulfate do not interfere with the formation of the tyrosine mercuric derivative within the limits prescribed by this experiment.*

### *II. Composition of the Tyrosine Mercury Compound. Preparation of the Compound.*

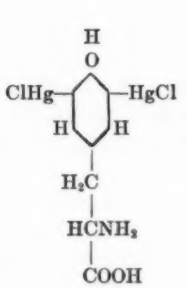
*Tyrosine.*—1.0 gm. was mixed with 750 cc. of water, 10 cc. of glacial acetic acid, and 35 gm. of mercuric acetate. The mixture was boiled for 5 minutes under a reflux condenser. The liquid was cooled in an ice bath, filtered from a small insoluble residue, and the clear filtrate treated with 75 gm. of sodium chloride. The mixture was centrifuged. The insoluble residue was washed ten times, by centrifugalization, with a 10 per cent solution of sodium chloride using about 50 cc. of the salt solution each time. The final washings did not contain mercury. The solid was then washed twice, rapidly, with distilled water to remove the sodium chloride. The perfectly white solid so obtained was dried *in vacuo* for 4 days over phosphorous pentoxide. It was then removed from the centrifuge tube, pulverized, transferred to a weighing bottle, and dried to constant weight *in vacuo*.

*Nitrogen.*—Of the compound, 0.3918 gm. was analyzed for nitrogen by the Kjeldahl process. The ammonia so liberated neutralized 2.96 cc. of 0.2 N HCl. This is equivalent to 0.021168 gm. of N per gm. of compound = *2.12 per cent N.*

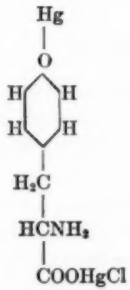
*Mercury.*—Of the compound, 1.1610 gm. were mixed with 175 cc. of normal sulfuric acid and boiled under a reflux condenser until the solid had dissolved completely. The hot liquid was saturated with hydrogen sulfide, the mixture heated for 30 minutes on the water bath, and filtered through a small, weighed filter paper. The thoroughly washed solid, dried to constant weight at 110°C., weighed 0.8228 gm. This is equivalent to 0.612 gm. of mercury per gm. of compound = *61.2 per cent Hg.*

*Chlorine.*—The filtrate from the HgS was concentrated on the water bath to a volume of about 150 cc. This was then diluted to exactly 200 cc. Of this solution two 50 cc. portions were titrated for chlorine with 0.1 N silver nitrate using the process of Volhard.[4] The entire 200 cc. of solution contained 0.1256 gm. of chloride ion which is equivalent to 0.1081 gm. of chlorine per gm. of compound = *10.81 per cent Cl.*

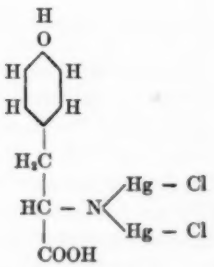
*Sodium.*—Of the above solution, 50 cc. were digested with concentrated sulfuric acid to destroy the tyrosine. The liquid was evaporated in a platinum crucible. A residue was not obtained. *Sodium was absent.* This was checked by repeatedly extracting 0.8 gm. of the dry mercury compound with water, which should dissolve any NaCl that might be present. The extracts left no residue on evaporation. These figures agree perfectly with the percentage composition of three possible formulæ.




I.




II.




III.

| | Theoretical values. *per cent* | Determined values. *per cent* |
|---|---|---|
| Nitrogen | 2.15 | 2.12 |
| Mercury | 61.6 | 61.2 |
| Chlorine | 10.9 | 10.81 |

*Location of the Mercury.*—This has already been adequately discussed in the introduction. Formula III represents the configuration of the molecule.

[4] Volhard, J., *Ann. Chem.*, 1878, cxc, 1.

### *III. The Quantitative Estimation of Histidine and Tyrosine in Protein.*

#### *The Method in Detail.*

Of the vacuum-dried protein, 1 to 3 gm. are mixed with 100 cc. of water and 15 cc. of 95 per cent sulfuric acid in a 300 cc. flask. The flask is attached to a reflux condenser by means of a ground glass joint. The mixture is boiled for 24 hours, cooled, transferred to a 3000 cc. flask, diluted to 1500 cc., heated on the water bath, and treated with a hot solution of 89 gm. of barium hydroxide in 500 cc. of water. The mixture is then adjusted with dilute solutions of barium hydroxide and sulfuric acid until it contains a slight excess of the sulfate ion. Digest for several hours and filter, while hot, on a Büchner funnel. The barium sulfate precipitate is thoroughly washed with hot water. The filtrate is transferred to a glass dish and concentrated on the steam bath.

#### *Silver Precipitation. Separation of Histidine and Tyrosine.*

The semisolid residue is transferred to a 1000 cc. flask with 50 cc. of water. Silver sulfate—600 cc. of an 0.8 per cent solution—is added and the liquid thoroughly mixed. It is then treated with a warm solution of 30 gm. of barium hydroxide in 100 cc. of water. The brown mixture is transferred, immediately, to an ice bath where it is allowed to settle for half an hour. Under these conditions the mixture remains brown; there is no noticeable deposition of metallic silver. The cold mixture is centrifuged. This effects a sharp separation in a cold medium in a minimum space of time. The clear, colorless, supernatant liquid is decanted into a 2000 cc. flask and immediately acidified with 20 per cent sulfuric acid.[5] The residue is washed once, by centrifugalization, with 50 cc. of a cold, saturated solution of barium hydroxide. The washings are added to the main bulk of the silver filtrate. This silver filtrate contains all of the tyrosine and is discussed later under the caption "Silver filtrate. Estimation of tyrosine."

[5] This liquid still contains a small amount of silver ion which invariably leads to the formation of a silver mirror and a loss of tyrosine as the solution warms up. Oxidation does not occur in the acidified liquid.

*The Silver Precipitate. Estimation of Histidine.*

The silver precipitate is transferred to a 500 cc. flask with 60 cc. of N sulfuric acid and 150 cc. of water. The mixture so obtained is treated with 3 cc. of 37 per cent HCl, agitated until it becomes homogeneous, and finally warmed on the steam bath, for 15 to 30 minutes, to complete the conversion of the silver compounds into silver chloride. When the conversion is complete, the silver chloride becomes unmistakably homogeneous and it is usually light colored. Filter (folded filter) into a 500 or 1000 cc. volumetric flask, wash the precipitate thoroughly with water until the washings are free from chlorides, neutralize the filtrate with 20 per cent NaOH, and dilute to volume.

Histidine is estimated, colorimetrically, in this liquid using the method of Koessler and Hanke.[6]

I have never encountered any difficulty in this fraction from interfering substances. The colors come up brilliantly and the values are practically identical with those previously obtained by the phosphotungstate method.[7] The phosphotungstate method involves adding a correction for the solubility of histidine phosphotungstate. Histidine silver seems to be quite insoluble; still the values are identical with those obtained with the phosphotungstate method *before* correcting for solubility. The divergence is never over 0.2 per cent.

It is well to remember that silver, and phosphotungstic acid, precipitate purines as well as histidine and that certain of the purines give a diazo reaction. Purines would, however, not be expected to occur in proteins other than nucleoproteins.

*Silver Filtrate. Estimation of Tyrosine.*

As has already been stated, the silver filtrate must be acidified with sulfuric acid before it gets warm to avoid oxidation of tyrosine by the silver which is invariably present in small amounts. A sufficient excess of sulfuric acid is added to cause the barium sulfate to settle readily. Normal hydrochloric acid is then added, drop by drop, until a precipitate of silver chloride is no longer obtained. The liquid should now give a faint test for chloride ion. It is heated on the steam bath and adjusted with barium hydroxide and sulfuric acid until a slight excess of sulfate ion is present.

[6] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 497, 521, 539.

[7] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1920, xliii, 527.

Digest for several hours, filter, transfer filtrate and washings to a glass dish, and concentrate on the steam bath.

*Precipitation of Tyrosine as Tyrosine Mercury Compound.*—The semisolid residue, which usually contains well defined crystals of tyrosine, is transferred to a 300 cc. flask with 75 cc. of water. Glacial acetic acid—1 cc.—and mercuric acetate—3.5 gm.—are added and the mixture either boiled for 10 minutes under a reflux condenser or heated for 1 hour on the steam bath without a reflux. A small amount of precipitate is always obtained. The mixture is cooled, treated with 7.5 gm. of sodium chloride, and placed in the ice chest for 2 hours. Transfer to a centrifuge tube with 20 cc. of a 10 per cent solution of sodium chloride. Centrifuge for 5 minutes at high speed. Decant and discard the clear supernatant liquid. The solid is washed once with 25 cc. of a 10 per cent solution of sodium chloride. The washed solid is transferred back quantitatively, to the boiling flask with 25 to 50 cc. of hot 20 per cent HCl. The mixture is heated on the steam bath for 30 minutes. Most, and frequently all, of the solid passes into solution. The liquid is treated with 50 cc. of water, saturated with hydrogen sulfide, heated on the water bath for 30 minutes, and filtered through a small folded filter. Filtrate and washings are collected in a glass dish and evaporated on the steam bath until all of the liquid has been removed. The pale brown crystalline residue is dissolved in water, diluted to 500 cc., and its tyrosine content estimated, colorimetrically.[1]

The histidine and tyrosine values obtained by this method for a number of proteins, are given in the following article.

*Note on the Estimation of Tyramine in Protein-Containing Mixtures.*

The mercury compound of tyramine is just as insoluble as that of tyrosine and it is formed under identical conditions. It probably has a chemical constitution analogous to that of the tyrosine compound. This compound can, therefore, be used as a means of separating tyramine from amino acids and other interfering substances that might occur in protein-containing mixtures. The method is briefly as follows:

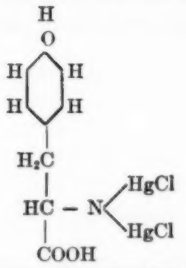
The mixture is hydrolyzed[2] with 20 per cent HCl and freed from water and acid by distillation *in vacuo*. Ammonia and humin

[2] This hydrolysis is always a laborious process because it dissolves so many things that could be eliminated by filtration at the outset. It seems,

are removed with barium hydroxide. Barium is removed with sulfuric acid. The concentrated aqueous solution of the residue is then treated with sodium hydroxide (final concentration of the alkali should be 30 per cent) and centrifuged. The clear supernatant liquid is exhaustively extracted with amyl alcohol. Amines are removed from the amyl alcohol with sulfuric acid. This solution, which contains all of the histamine and tyramine and small amounts of the corresponding amino acids, is freed from sulfuric acid, concentrated, realkalinized, and reextracted with amyl alcohol. This time the amino acids remain, quantitatively, in the aqueous layer. The amines are in the amyl alcohol, from which they are extracted with normal sulfuric acid. The liquid is neutralized with barium hydroxide, the filtrate from the barium sulfate concentrated and subjected to a silver precipitation. This precipitates the histamine as silver salt and leaves tyramine in solution. Histamine is then estimated as previously described.[9] Tyramine is precipitated with mercuric acetate and finally estimated colorimetrically.

SUMMARY.

When tyrosine is boiled with mercuric acetate under suitable conditions, and the resulting clear solution treated with sodium chloride, a white solid is obtained that has the formula:




however, to be essential because aqueous or alcoholic extracts of feces or tissue, in which one would expect the amines to dissolve, do not always contain the amines. The latter are largely attached to the insoluble matter either by adsorption or in a more stable form of combination. To obtain a reasonably quantitative yield of histamine or tyramine it is, at least for the present, necessary to decompose the insoluble matter as far as possible by hydrolysis.

[9] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1920, xliii, 543.

This compound is very insoluble in water and in a mixture of amino acids. It can serve, therefore, as a means of separating tyrosine more or less completely from the other amino acids that are formed by the acid hydrolysis of a protein. The solid can be dissolved in dilute sulfuric acid or in 20 per cent HCl. Mercury can be quantitatively removed from the compound by passing hydrogen sulfide into its suspension in water or its solution in acids. The resulting solution contains all but a mere trace of the tyrosine that was originally present in the substance under investigation and not much of anything else. The exact concentration of tyrosine can then be most accurately estimated by the process of Hanke and Koessler.[1] Protein hydrolysates contain amino acids that are, to a small extent, precipitated with the tyrosine. With the exception of histidine, these amino acids in such small quantities do not interfere with the colorimetric estimation of tyrosine. The complete analytical process is, briefly, as follows: The protein is hydrolyzed with sulfuric acid. Histidine is precipitated as silver complex and its amount estimated colorimetrically. The filtrate from the histidine silver is boiled with mercuric acetate and treated with sodium chloride which precipitates tyrosino-mercuric chloride. The precipitate is dissolved in 20 per cent HCl, freed from mercury with hydrogen sulfide, and the tyrosine estimated in the filtrate from the mercuric sulfide.[1]

A method for estimating tyramine in protein-containing matter is briefly outlined.

# THE HISTIDINE AND TYROSINE CONTENT OF A NUMBER OF PROTEINS.

By MILTON T. HANKE.

*(From the Otho S. A. Sprague Memorial Institute and the Department of Pathology, University of Chicago, Chicago.)*

(Received for publication, September 12, 1925.)

Histidine has been determined, in the past, by the original method of Kossel and Kutscher,[1] by one of the numerous modifications of this method,[2] and by the group method of Van Slyke.[3] In 1920, Dr. Koessler and I published a method[4] that has the advantages of being simple, rapid, and accurate with small quantities—1 to 3 gm.—of protein. The method described in the preceding communication is just as accurate, is, if anything, simpler, and has the added advantage that the same sample of material can also be used for the estimation of tyrosine.

Tyrosine has, in the past, been determined gravimetrically and colorimetrically. The colorimetric processes have at least one thing in common; namely, they all give results that are decidedly higher than those obtained gravimetrically. A properly conducted gravimetric procedure will, of course, give minimum values. These may or may not be the true values depending upon the solvent action of the other constituents of the crystallization medium. This fact has led some writers to accept the higher figure[5] in preference to the gravimetric figure.

[1] Kossel, A., and Kutscher, F., *Z. physiol. Chem.*, 1900–01, xxxi, 165.

[2] Kossel, A., and Patten, A. J., *Z. physiol. Chem.*, 1903, xxxviii, 39. Steudel, H., *Z. physiol. Chem.*, 1903, xxxvii, 219. Kossel, A., and Pringle, H., *Z. physiol. Chem.*, 1906, xlix, 318. Osborne, T. B., Leavenworth, C. S., and Brautlecht, C. A., *Am. J. Physiol.*, 1908–09, xxiii, 180.

[3] Van Slyke, D. D., *J. Biol. Chem.*, 1911–12, x, 15.

[4] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1920, xliii, 527.

[5] Sherman, H. C., Food products, New York, 2nd edition, 1924, 78 (footnote).

The colorimetric processes are, however, not specific for tyrosine. The reagent of Folin and Denis[6] is readily reduced by substances other than tyrosine, and bromine is, of course, reduced or absorbed by many organic compounds. The very careful work of Fürth and his collaborators[7] has, I believe effectively demonstrated the unreliability of the above two methods. The quantitative Millon procedure advocated by O. Fürth and A. Fischer[7] is not reliable, as I shall try to show later, because the procedure is sensitive to slight and uncontrollable variations in the composition of the final reaction liquid. The procedure described in the preceding communication is, in a way, similar to the gravimetric process because it first removes the tyrosine, as mercury compound, from the bulk of amino acids. The mercury compound of tyrosine is, however, far less soluble than tyrosine itself, and its precipitation does not seem to be hindered by the presence of amino acids. The colorimetric procedure finally employed for the determination of tyrosine is direct and characteristic for tyrosine.[8]

The values obtained with this new method and a comparison of these with the results obtained by other methods are summarized in Table I.

## DISCUSSION OF RESULTS.

Table I reveals rather strikingly that the tyrosine figures obtained by the new method are in fairly close agreement with those obtained by the gravimetric method. Thus for casein, squash seed globulin, gliadin, and zein, the results are identical. In other cases—gelatin, egg albumin, hordein, and secalin—the values are slightly higher than those obtained gravimetrically, which is to be expected. The values obtained are invariably much lower than those obtained by the method of Folin and Denis,[6] the bromine addition process, and the Millon reaction.[7]

The very careful studies of Fürth and his collaborators[7] have

[6] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 245.

[7] Fürth, O., *Biochem. Z.*, 1924, cxlvi, 259. Fürth, O., and Fischer, A., *Biochem. Z.*, 1924, cliv, 1.

[8] A similar color is also obtained with tyramine and with certain volatile or labile enolic compounds; but none of these would be expected to occur in a protein and all but the tyramine would be lost or destroyed by the outlined procedure.

TABLE I.

| Protein. | Observed amino acid content. | | Amino acid content. Other investigators. (Top figures are gravimetric.) | | |
|---|---|---|---|---|---|
| | Histidine. | Tyrosine. | Histidine. | Tyrosine. | |
| | *per cent* | *per cent* | *per cent* | *per cent* | |
| Gelatin, commercial. | 0.54 | 0.25 | 0.40 | 0.00 | Fischer, E., Levene, P. A., and Aders, R. H., *Z. physiol. Chem.*, 1902, xxxv, 70. |
| | 0.52 | 0.26 | | | |
| | | | | 1.20 | Fürth, O., and Fischer, A., *Biochem. Z.*, 1924, cliv, 1. |
| Casein (cow), highly purified. | 2.63 | 4.5 | 2.6 | 4.5 | Fischer, E., *Z. physiol. Chem.*, 1901, xxxiii, 151. |
| | 2.60 | 4.5 | | | |
| | | | | 6.5 | Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 247. |
| | | | | 4.7–5.3 | (Bromine process.) Fürth, O., and Fleischmann, W., *Biochem. Z.*, 1922, cxxvii, 137. |
| | | | | 5.9–8.0 | (Millon reaction.) Fürth and Fischer, *loc. cit.* |
| Egg albumin, crystalline. | 2.28 | 2.35 | 1.71 | 1.77 | Osborne, T. B., Jones, D. B., and Leavenworth, C. S., *Am. J. Physiol.*, 1909, xxiv, 252. |
| | 2.31 | 2.35 | | | |
| | | | | 5.0 | Folin and Denis, *loc. cit.* |
| Squash seed globulin.* | 2.25 | 3.03 | 2.63 | 3.07 | Osborne, T. B., and Clapp, S. H., *Am. J. Physiol.*, 1907, xix, 475. |
| | 2.28 | 3.06 | | | |
| | | | | 4.9 | Folín and Denis, *loc. cit.* |
| Gliadin.* | 2.08 | 2.31 | 0.61 | 1.20 | Osborne and Clapp, *loc. cit.* |
| | 2.10 | 2.39 | 1.20 | 2.39 | Abherhalden, E., and Samuely, F., *Z. physiol. Chem.*, 1905, xliv, 276. |
| | | | | 3.3 | Folin and Denis, *loc. cit.* |

TABLE I—*Concluded.*

| Protein. | Observed amino acid content. | | Amino acid content. Other investigators. (Top figures are gravimetric.) | | |
|---|---|---|---|---|---|
| | Histidine. | Tyrosine. | Histidine. | Tyrosine. | |
| | *per cent* | *per cent* | *per cent* | *per cent* | |
| Hordein.† | 0.98 | 2.37 | 1.28 | 1.67 | Osborne and Clapp, *loc. cit.* |
| | 0.98 | 2.50 | 0.51 | 4.00 | Kleinschmidt, A., *Z. physiol. Chem.*, 1907, liv, 110. |
| | | | | 4.7 | Folin and Denis, *loc. cit.* |
| Zein.* | 1.25 | 3.66 | 0.82 | 3.55 | Osborne, T. B., and Liddle, L. M., *Am. J. Physiol.*, 1910, xxvi, 295. |
| | | | 0.82 | 3.19 | Osborne, T. B., and Jones, D. B., *Am. J. Physiol.*, 1910, xxvi, 212. |
| | | | | 5.5 | Folin and Denis, *loc. cit.* |
| Secalin.† | 1.23 | 1.37 | 0.39 | 1.19 | Osborne, T. B., and Clapp, S. H., *Am. J. Physiol.*, 1907–08, xx, 494. |
| Sativin.† | 0.74 | 1.56 | | | |
| Sorghumin.† | 0.51 | 2.3 | | | |
| | 0.51 | 2.25 | | | |
| Fibrin (sheep).† | 2.18 | 3.33 | | | Histidine and tyrosine values for these four fibrins are not recorded in the literature. Horse fibrin has been found to contain 3.5 per cent of tyrosine. Histidine was not determined. Abderhalden, E., and Voitinovici, A., *Z. physiol. Chem.*, 1907, lii, 371–374. |
| | | 3.30 | | | |
| Fibrin (swine).† | 2.27 | 3.45 | | | |
| Fibrin (Cattle A).† | 2.05 | 3.52 | | | |
| Fibrin (Cattle B).† | 2.06 | 3.45 | | | |

The proteins marked * and † were kindly supplied by T. B. Osborne and Ross Aiken Gortner respectively for which I wish to express my sincere appreciation.

effectively demonstrated the unreliability of the method of Folin and Denis and of the bromine absorption process. Estimation of tyrosine by the Millon reaction, as outlined by O. Fürth and A. Fischer[7] seems also to be rather unreliable if I may interpret

their figures. They find, for example, that by increasing the amount of free sulfuric acid in both the protein hydrolysate and in the standard tyrosine comparison liquid, values for casein varying from 5.9 to 8.0 per cent are obtained, a divergence of 2.1 per cent. Merely changing the concentration of tyrosine in the standard comparison liquid from 0.05 to 0.033 per cent introduces a change in the tyrosine value of casein of, in one series, from 6.0 to 7.0 per cent. Just which one of these conditions, if any, gives the correct figure? Merely adding them together and taking the mean value, as they have done, can surely not lead to a reliable figure.

I have also conducted experiments, some 2 years ago, in an attempt to use the Millon reaction for estimating the tyrosine content of solutions and I found that the *absolute* colors so obtained were very markedly dependent upon the concentration of tyrosine, of free acid, of mercury, and of nitrous acid. These factors, with the exception of the nitrous acid, are also mentioned by O. Fürth and A. Fischer. With all of these factors carefully controlled under ideal conditions I still found it possible to make errors as great as 10 per cent in estimating the tyrosine content of pure solutions of tyrosine. When we go from a pure solution to one containing a mixture of amino acids the difficulties must, necessarily, be increased because the other amino acids can change the acidity, the mercury ion content, and, most particularly, the concentration of nitrous acid. This factor, the amino acid content, is beyond our control and is, necessarily, different for most proteins. It is difficult for me to understand, then, how such a method can give a reliable tyrosine figure.

SUMMARY.

By means of the method described in the preceding article, the histidine and tyrosine content of a number of proteins has been determined. The mean values obtained are as follows. (The first figure is the per cent of histidine, the second the per cent of tyrosine.) Gelatin 0.53, 0.25; casein 2.61, 4.5; crystalline egg albumin 2.3, 2.35; squash seed globulin 2.26, 3.05; gliadin 2.1, 2.35; hordein 0.98, 2.43; zein 1.25, 3.66; secalin 1.23, 1.37; sativin 0.74, 1.56; sorghumin 0.51, 2.3; fibrin (sheep) 2.18, 3.3; fibrin (swine) 2.27, 3.45; fibrin (cattle) 2.05, 3.5.

# A CONTINUOUS DIALYSIS OR EXTRACTION APPARATUS WHICH OPERATES AT REDUCED PRESSURE WITH A CONSTANT VOLUME OF LIQUID.

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(Received for publication, September 12, 1925.)

## INTRODUCTION.

Although many types of extraction and dialysis apparatus have been devised, experimental problems still present themselves for which the existing kinds of apparatus are not entirely satisfactory. This is, perhaps, more certainly true of dialysis than of extraction. Dialysis to remove salts and other crystalloids is simple enough when the electrolytes contained in the tap water do not interfere with the success of the experiment. It becomes more difficult when an exhaustive dialysis against distilled water is required and becomes laborious when both dialysate and diffusate are to be collected.

The apparatus to be described is so designed that the liquid volume of the system remains constant. The absolute volume of the diffusate is solely dependent upon the size of the apparatus, largely upon the size of the diffusion chamber. This volume can, therefore, be controlled at will, which is highly desirable when the diffusate is to be subjected to analysis.

The dialysate is constantly surrounded by freshly distilled solvent at room temperature. If so desired, the diffusion chamber can be immersed in a bath which can be kept at any desired temperature below that of the boiling point of the solvent at the operating pressure. Dialysis or extraction can, therefore, be carried out at any desired temperature.

The dialysate is agitated constantly, which greatly increases the speed of dialysis.

Both dialysate and diffusate can be kept fairly sterile by the use of suitable antiseptics. Phenol is excellent when its use does not interfere with the outcome of the experiment because, being volatile with water vapor, it is ever present in all parts of the apparatus. A layer of toluene on the dialysate helps not only to keep it sterile but, by diffusing into the diffusate, also sterilizes the latter. This is particularly effective when all of the air intake tubes are connected to wash bottles containing toluene. The choice of these or other antiseptics will depend entirely upon the nature of the experiment.

After a preliminary adjustment, which is neither difficult nor time-consuming, the apparatus functions automatically and can be operated for days with practically no attention. Slight fluctuations in the operating pressure, which are unavoidable when a water aspirator is used, do not interfere with the operation of the apparatus.

*Detailed Description of the Apparatus.*

The accompanying diagram illustrates the construction of the apparatus. Flask *A* is a round bottom Pyrex flask of suitable capacity. It is suspended in a constant-level, electrically heated, water bath. An amount of distilled water at least 100 cc. in excess of that required to fill the *empty* dialysis chamber to the outlet *F*, is introduced. The special condenser *C* must have at least seven and preferably eight condensing bulbs; otherwise some of the vapor will escape condensation and the water supply will have to be replenished from time to time. The dialysis chamber is merely a large glass tube with an overflow outlet on one side. For a chamber 23 cm. high and 5 cm. in diameter, the outlet tube should be located about 10 cm. from the top. The top of this tube is sealed with a large rubber stopper. To keep this stopper from being drawn into the apparatus when the air is exhausted, a circular line of indentations is made 1 cm. from the open end of the tube. The dialysis sack can be of any suitable material. I have used both parchment and the so called fish skin condoms. The open end of the sack is slipped over a specially grooved, two-hole stopper, as illustrated, and held in place with a rubber band. The material to be dialyzed is then introduced into the sack and the whole is slipped on the tube *J* which is a narrow tube except

near the top where it widens out to act as a support for the stopper and sack.

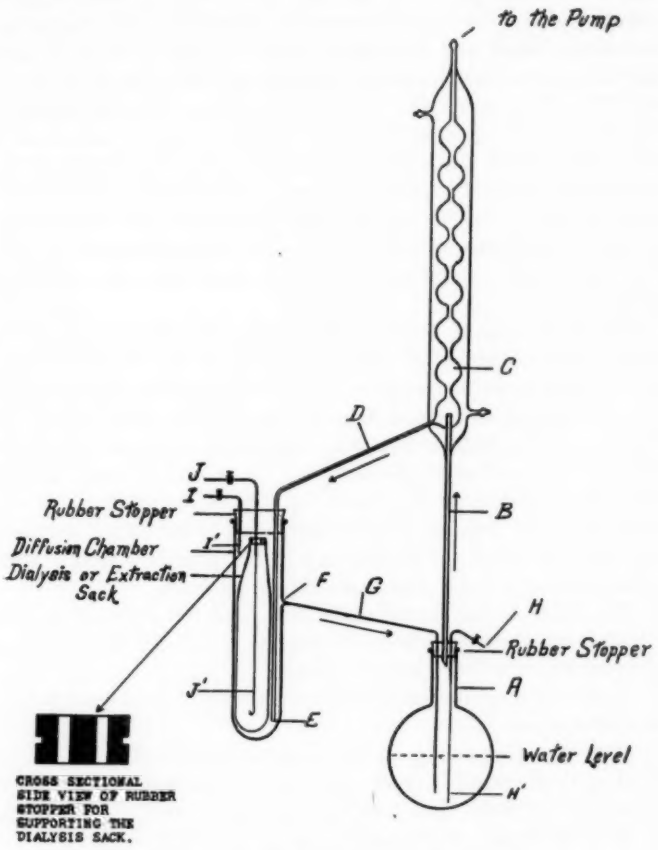


FIG. 1. Vacuum dialysis and extraction apparatus.

Rubber connections are introduced, at *B*, *D*, and *G* to facilitate in assembling the apparatus and to introduce a very essential element of flexibility which reduces the strain on the glass parts.

After the apparatus has been assembled with the dialysate in

place, stop-cocks *H*, *I*, and *J* are closed and the aspirator pump turned on. When the apparatus is completely exhausted, stop-cock *J* is opened cautiously until air bubbles can be seen rising at about 1 second intervals through the dialysate. This keeps the dialysate stirred and facilitates dialysis. Stop-cock *H* is then partially opened until bubbles can be seen rising about the capillary *H'*. This air intake is essential to insure smooth boiling of the liquid contained in flask *A*. Finally connect stop-cock *I* to a small, tight, wash bottle by means of a tight rubber hose. Open until the bubbles rise in the wash bottle at the rate of about 1 per second. This air intake, together with the one that is used to stir the dialysate, supplies the pressure that is necessary to force the liquid from the dialysis chamber back into the ebullition flask.

The electric heater is now turned on and the temperature of the water bath controlled so that the liquid, in the ebullition flask, boils. The vapor rises in tube *B* and is condensed in the condenser *C*. The liquid so formed flows down through tube *D* into the bottom of the dialysis chamber. After the dialysis chamber is filled to the overflow *F*, the diffusate flows back through tube *G* into the ebullition flask *A* and the process is repeated. Stop-cocks *H* and *I* may have to be readjusted to insure rapid boiling and perfect return of the diffusate to the ebullition flask. Once adjusted they usually require no further attention.

The dialysate may, occasionally, foam. A few drops of caprylic alcohol can then be introduced through stop-cock *J* which will prevent foaming for at least 24 hours.

A 24 to 48 hour dialysis is usually sufficient to remove all diffusible substances.

After the dialysis has been completed, shut off the stop-cock leading to the pump. Open stop-cock *I* cautiously (so as not to drive the diffusate back into the condenser) until the apparatus is filled with air. Remove the water bath and cut the rubber connection at *G*. The ebullition flask and the dialysis chamber can now be removed without disturbing the rest of the apparatus.

Although the above description is rather specific for the apparatus now in use in this laboratory, the apparatus will function equally well when modified in a large number of ways. It can be made of practically any size and it need not, of course, be constructed of

glass. It will serve for extraction as well as dialysis. Extraction or dialysis can be carried out at any desired temperature below the boiling point of the liquid at the operating pressure. This operating pressure is also subject to variation within wide limits. It should also be possible to use practically any liquid if suitable changes are made in the operating pressure and in the mode of condensation.

# AN ALCOHOL-SOLUBLE PROTEIN ISOLATED FROM POLISHED RICE.*

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Of all the seeds of cereals thus far examined, rice is the only one from which an alcohol-soluble protein or prolamine has not been isolated in considerable quantity. Most of the prolamines are readily soluble in 60 to 70 per cent alcohol at room temperature. The seeds of kafir[1,2] and of sorghum,[2] however, yield an alcohol-soluble protein only when extracted with *hot* 70 to 80 per cent alcohol. The proteins thus obtained, however, possess the characteristics of the prolamines including a relatively high amide nitrogen content.

Comparatively little work has been conducted on the proteins of rice. Rosenheim and Kajiura[3] studied the proteins of rice and isolated a globulin, an albumin, and a glutelin. Concerning an alcohol-soluble protein they state, "The absence of any protein soluble in alcohol is a characteristic feature of rice. From three kilogrammes of powdered rice we only obtained on the average 0.4 grammes of a nitrogenous substance soluble in dilute alcohol, and it is doubtful whether this is really or wholly a protein." About the same time Suzuki, Yoshimura, and Fuji[4] found that a small percentage of the nitrogen of polished rice is soluble in 60 per cent alcohol, but they gave no data to show whether or not

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[1] Johns, C. O., and Brewster, J. F., *J. Biol. Chem.*, 1916–17, xxviii, 59.

[2] Hoffman, W. F., and Gortner, R. A., Colloid symposium monograph, New York, 1924, ii, Chapter 20.

[3] Rosenheim, O., and Kajiura, S., *J. Physiol.*, 1907–08, xxxvi, p. liv.

[4] Suzuki, U., Yoshimura, K., and Fuji, S., *J. Coll. Agric. Tokyo*, 1909, i, 77.

this nitrogen was from an alcohol-soluble protein. Osborne, Van Slyke, Leavenworth, and Vinograd[5] isolated a very small amount of an alcohol-soluble protein from rice. They obtained this by dissolving all of the protein of rice in dilute sodium hydroxide, and precipitating the protein by the addition of acetic acid. The protein thus obtained was extracted with hot 80 per cent alcohol. The alcohol extracts were concentrated and found to contain a small amount of protein "similar to gliadin from wheat in its solubility." The amount obtained was too little for analysis, and they were not able to determine whether it was an original constituent of the seed or a derived product.

To determine whether or not the alcohol-soluble protein of rice obtained by Osborne *et al.* possesses the physical and chemical characteristics of the prolamines, an attempt was made to prepare this protein in a sufficient quatity for analysis.

6.5 kilos of commercial polished rice were ground to a fine meal and extracted several times with hot 70 per cent alcohol. This alcoholic solution was filtered and the clear filtrate concentrated in vacuum. Due to excessive foaming, the solution could not be concentrated to a sirup, but after most of the alcohol had been removed the remainder of the solution was evaporated to dryness at about 40°C. in an open dish. The product thus obtained was redissolved in hot 70 per cent alcohol and the solution filtered. This alcoholic solution was then placed in a collodion bag and dialyzed against distilled water. This removed the alcohol and all water-soluble substances. After the major portion of the alcohol had been removed, the protein separated in a white flocculent mass. Part of the water was removed by evaporation through the collodion bag[6] after which the precipitate and the remaining water were transferred to an open dish and the water evaporated off at about 40°C. The resulting product after grinding was a gray-brown powder. This was extracted with absolute alcohol followed by ether. These solvents removed a small amount of oil and coloring matter. The yield of protein was 7.5 gm. of the dried product or 0.12 per cent of the polished rice.

[5] Osborne, T. B., Van Slyke, D. D., Leavenworth, C. S., and Vinograd, M., *J. Biol. Chem.*, 1915, xxii, 259.

[6] The "pervaporation" of Kober, P. A., *J. Am. Chem. Soc.*, 1917, xxxix, 944.

The results of the elementary analysis show it to contain on the ash- and moisture-free basis:

| | Prolamine (rice). | Gliadin[7] (wheat). |
|---|---|---|
| Carbon | 55.92 | 52.72 |
| Hydrogen | 6.49 | 6.86 |
| Nitrogen | 16.22 | 17.66 |
| Sulfur | 1.14 | 1.14 |

These results show that rice prolamine contains a smaller amount of nitrogen and more carbon than does the wheat prolamine. However, the analysis for the rice protein does not differ essentially from those for the prolamines of maize, kafir, etc.

TABLE I.

*The Nitrogen Distribution of the Alcohol-Soluble Protein of Rice as Determined by the Van Slyke Method, Expressed in Per Cent of Total Nitrogen.*

| | | Rice prolamine. | | | Rice[5] glutelin (oryzenin). | Wheat gliadin.[5] |
|---|---|---|---|---|---|---|
| | | A | B | Average. | | |
| Ammonia nitrogen | | 12.43 | 12.46 | 12.45 | 11.33 | 24.61 |
| Humin nitrogen | Insoluble | 1.69 | 1.61 | 1.65 | 1.59 | 0.52 |
| | Soluble | 1.77 | 1.58 | 1.68 | | 0.35 |
| Basic nitrogen | Arginine | 13.13 | 13.29 | 13.21 | 17.69 | 6.38 |
| | Histidine | 6.25 | 6.23 | 6.24 | 5.39 | 5.41 |
| | Cystine | 1.31 | 1.34 | 1.33 | 0.88 | 1.68 |
| | Lysine | 1.72 | 1.74 | 1.73 | 4.90 | 0.57 |
| N in filtrate from bases | Amino | 53.92 | 53.26 | 53.59 | 52.13 | 53.49 |
| | Non-amino | 7.05 | 7.59 | 7.32 | 5.28 | 6.14 |
| Total | | 99.27 | 99.10 | 99.20 | 99.19 | 99.15 |

When subjected to analysis according to the Van Slyke method of nitrogen distribution, the results, Table I, show that this protein differs from the typical prolamines, using gliadin from wheat as an example, neither does it resemble the rice glutenin. It does not contain the high percentage of ammonia nitrogen which

[7] Osborne, T. B., and Harris, I. F., *Am. J. Physiol.*, 1906–07, xvii, 223.

is typical of the prolamines; it contains more basic nitrogen than any prolamine heretofore isolated, but only a small amount of the basic nitrogen is lysine. The arginine content is much higher than that of any known prolamine but is lower than the values reported for the associated protein, oryzenin.

These results show that rice contains but a trace of a protein soluble in hot alcohol. This protein resembles the prolamines of kafir and sorghum in solubility but does not contain the large amount of amide nitrogen which has been considered characteristic of prolamines. It is not probable that this protein is part of the rice glutelin inasmuch as no more than the amount obtained could be extracted, *i.e.*, after the first two or three extractions no additional protein was removed by the hot alcohol. Neither is it probable that it is not an original constituent of the seed because the rice had received no treatment other than the polishing and grinding before extraction.

# THE RELATION BETWEEN COLORIMETRIC READING AND TRUE pH OF SERUM OR PLASMA.

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(Received for publication, August 3, 1925.)

Because of its simplicity and the economy of material and time the determination of the pH of serum or plasma colorimetrically has attracted the efforts of several investigators. The methods in chief use at present are those in which the protein error is eliminated by dialysis, as in the method of Dale and Evans or Lindhard, and those in which the protein error is diminished by dilution and then corrected for, as in the method of Cullen and that of Hastings and Sendroy. In any method precautions must be introduced to handle the blood and plasma (or serum) without loss of $CO_2$.

Some confusion in the discussions of colorimetric pH can be traced to the use of a pH notation to indicate the indicator color. Thus we speak of a colorimetric reading of 7.40 at 20° meaning the color that would be produced when the indicator is added to a phosphate buffer with a $pH_{20°} = 7.40$. This notation becomes especially confusing when we deal with different temperatures. We have introduced Fig. 1 to express graphically the relation between the color of phenol red standards prepared according to the method of Hastings and Sendroy and phenol red standards prepared from Sörensen's phosphate buffers with the pH of the latter measured at 38° and 20° electrometrically. The electrometric pH measurements are standardized against N/10 HCl, calling its pH at both 20° and 38°, pH = 1.080 (see Cullen, Keeler, and Robinson, 1925). Fig. 1 is only a modification of Fig. 1 of Hastings and Sendroy. We have altered the scale of the abscissæ so that the isotherms become straight lines. We have added lines indicating the pH and the phenol red color in Sörensen's phosphate

buffers at 20° and 38°. The abscissæ representing color are given in four notations: (1) the log $\frac{R}{Y}$, which is the logarithm of the ratio of red to yellow component in the standard; (2) red per cent, which is per cent of indicator in the red form; (3) $[pH]_{38°}$, which is the color given by phenol red in phosphate buffer having that

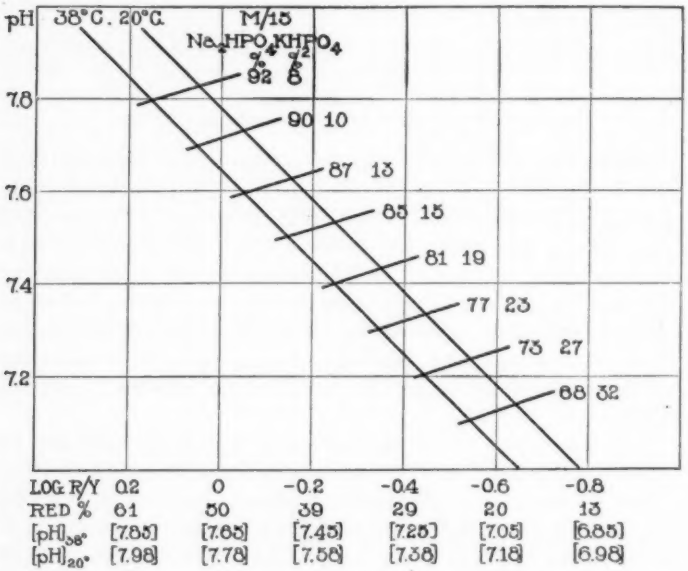


FIG. 1. Showing relation of phenol red indicator color in saline buffer solutions to true pH and to Sörensen's phosphate mixtures at varying temperatures.

Ordinates give temperature in °C.

$\frac{R}{Y}$ = ratio of red dye solution to yellow dye solution (bicolor standards prepared according to Hastings and Sendroy).

$[pH]_{20°}$ and $[pH]_{38°}$ conventional notation for color of indicator mixtures.

pH at 38°; (4) $[pH]_{20°}$ which is the color given by phenol red in phosphate buffer having that pH at 20°. Throughout this paper we will employ a bracketed symbol with a subscript of either 38° or 20° to designate indicator color and an unbracketed symbol to designate pH. It will be understood from Fig. 1 that the same phenol red color is denoted by $[7.40]_{20°}$ and $[7.27]_{38°}$. This

is essentially the same notation as that followed by Hastings and Sendroy in designating their bicolor standards. This notation is adopted simply for convenience inasmuch as a phosphate buffer at 20° with a $pH_{20°} = 7.40$ gives the same indicator color with phenol red as the different phosphate buffer which at 38° has with our pH standardization a $pH_{38°} = 7.27$. It will be seen from the graph that the same phosphate buffer which at 20° has a $pH_{20°} = 7.40$ and gives an indicator color of $[7.40]_{20°}$, has at 38° a $pH_{38°} = 7.3$, and gives an indicator color of $[7.37]_{38°}$ or $[7.50]_{20°}$. Fig. 1 serves also, therefore, as an expression of the relation of our standardization of pH at varying temperature to Sörensen's phosphate buffers.

That there is an effect of salt and protein upon indicator color is well recognized. We have found that substituting other anions or cations in a saline-buffer-serum mixture while keeping the true pH of the solution constant produces much greater change in the color of phenol red than occurs upon substituting other anions or cations at constant pH in a saline-buffer mixture without serum. The addition of a small amount of serum to a phosphate buffer containing indicator results in a change of indicator color [pH], even when the experiment is so conducted that there is no change in the true pH of the solution. This is due to the so called protein error. That is, in the presence of serum, the relation of the color scale to pH is not shown in Fig. 1. The effect of varying dilution and of varying the nature of the salts in the diluting fluid upon the color reading is only in part due to resulting variations in pH; it is largely due to variations in the color [pH] for the same pH. Further studies of this well known phenomenon are in progress.

In developing his colorimetric method for plasma pH Cullen (1922) read the color of the diluted serum at 20° against a color scale identical with our scale $[pH]_{20°}$ and also determined electrometrically the true $pH_{38°}$ of the undiluted serum. This difference $[pH]_{20°} - pH_{38°}$ he called his C correction.

Hastings and Sendroy reported that if the diluted plasma or serum be read colorimetrically at 38° and the color expressed in the $[pH]_{38°}$ scale then in all the sera they studied $[pH]_{38°} - pH_{38°} = 0$. This has not been consistently our experience. The correction $([pH]_{38°} - pH_{38°})$ we have designated as the H correction: the difference between color reading of diluted serum (or plasma) at 38° and the true pH of undiluted serum (or plasma) at 38°.

In this paper we have collected a series of our observations made on serum or plasma of dog, sheep, and man to show the values for the C and H corrections.

*Methods.*

Blood was drawn from a vein or from the heart under oil and either defibrinated under oil or oxalated by having coated the receiving vessel with neutral potassium oxalate in amount sufficient to make the final concentration 0.3 per cent. Equilibration and separation of serum or plasma were done by the technique described by Austin, Cullen, Hastings, McLean, Peters, and Van Slyke (1922), using the second saturation method.

Electrometric pH was determined by the method described by Cullen, Keeler, and Robinson (1925), standardizing our readings against N/10 HCl and calling its pH = 1.080 at both 20°C. and 38°C.

Colorimetric [pH] was determined at 20° and at 38° by the methods of Cullen (1922) and of Hastings and Sendroy (1924) using the standards suggested by the latter authors and expressing the results in the notation indicated in Fig. 1 of this paper. Gasometric pH was calculated by the Henderson-Hasselbalch equation

$$pH = pK' + \log \frac{[CO_2]_s - \alpha p_{CO_2}}{\alpha p_{CO_2}}$$

where pK′ is taken as 6.10 at 38° and 6.19 at 20° (see Cullen, Keeler, and Robinson, 1925) and where

$\alpha$ = 0.975 $\alpha$ water (Bohr's value),
$[CO_2]_s$ = total $CO_2$ concentration in serum or plasma.

Throughout this paper brackets around pH values indicate color readings. Other values enclosed in brackets indicate concentrations in mM per liter.

In the table and text "serum as drawn" indicates that the [pH] was read without equlibration. "Separated serum (or plasma)" indicates that the serum (or plasma) was separated from the cells and subsequently equilibrated and its [pH] and $[CO_2]$ determined. True serum (or plasma) indicates that the whole blood was equilibrated and then its serum (or plasma) separated without loss of $CO_2$ and its [pH] and $[CO_2]$ determined.

RESULTS.

The results are tabulated in Table I.

The true pH has been determined in some instances electrometrically and in other instances gasometrically by equilibrating either the separated serum (or plasma) or the whole blood and then separating its true serum or plasma and calculating pH by the Henderson-Hasselbalch equation. A colorimetric [pH] determination was then made on each serum or plasma both at 20° and at 38° the results being expressed in the $[pH]_{20°}$ and $[pH]_{38°}$ scales respectively (see Fig. 1 and Table I, Columns 7 and 6). In Column 8 is given the H correction ($[pH]_{38°} - pH_{38°}$) or when the $pH_{20°}$ of the undiluted serum was known we give the analogous ($[pH]_{20°} - pH_{20°}$) difference. In Column 9 is given Cullen's C correction ($[pH]_{20°} - pH_{38°}$).

It will be seen that the agreement between gasometric pH and electrometric pH where both were done is within 0.03. This has been repeatedly confirmed in our laboratory and we feel justified in assuming that our gasometric pH values represent true pH values within this error.

The variations in the H correction ($[pH]_{38°} - pH_{38°}$) are for the dog from $-0.03$ to $+0.17$ and, furthermore, this range of variation may occur in the same animal when examined on different days. Usually, pronounced variations in this H correction have been associated with severe bleedings, the development of a distemper, or some other experimental or spontaneous change in the animal's condition. We have not as yet been able to correlate the variations with any one factor. The variations in the dog of ($[pH]_{20°} - pH_{20°}$) are from 0.00 to $+0.11$. In the sheep the H correction has varied from $-0.06$ to $+0.03$ and ($[pH]_{20°} - pH_{20°}$) from $-0.11$ to $-0.08$. In pooled rat serum the H correction varied from $+0.02$ to $+0.06$. In nine human patients with miscellaneous conditions the H correction varied from $-0.02$ to $+0.20$. The highest of these was checked by repetition 3 days later when a value of $+0.18$ was obtained.

We have concluded from these observations that we cannot rely upon the colorimetric reading of a serum or plasma by the technique of Hastings and Sendroy to give us the true pH directly from the color reading. Some method of determining the H correction for each serum must be employed.

TABLE I.

*Dog Serum or Plasma (Equilibrated at 38°C. and 40 Mm. $P_{CO_2}$ except When Otherwise Specified).*

| Source. | Date. | Material.* | $pH_{38°}$ | | Indicator color. | | H correction. $[pH]_{38°} - pH_{38°}$ | C correction. $[pH]_{38°} - pH_{38°}$ |
|---|---|---|---|---|---|---|---|---|
| | | | Gasometric. | Electrometric. | $[pH]_{38°}$ | $[pH]_{38°}$ | | |
| | *1925* | | | | | | | |
| Dog 27 | Jan. 6 | Serum as drawn. | | 7.35 | [7.52] | [7.81] | +0.17 | +0.46 |
| | " 7 | " " " | | 7.38 | [7.53] | [7.83] | +0.15 | +0.45 |
| | " 9 | " " " | | 7.35 | [7.52] | [7.82] | +0.17 | +0.47 |
| | " 14 | Separated serum 38°, 90 mm. | 7.08 | 7.07 | [7.25] | [7.41] | +0.17 | +0.34 |
| | | " " 38°, 25 " | 7.58 | 7.58 | [7.64] | [7.87] | +0.06 | +0.29 |
| | | Serum as drawn. | | | [7.50] | [7.73] | | |
| | Mar. 18 | Separated serum 38°. | 7.05 | | [7.05] | [7.21] | −0.01 | +0.16 |
| | | | 7.31 | | [7.40] | [7.60] | +0.09 | +0.29 |
| | | | 7.60 | | [7.63] | [7.86] | +0.03 | +0.26 |
| | Apr. 9 | Separated serum 38°. | 7.25 | | [7.41] | [7.63] | +0.16 | +0.38 |
| | " 14 | " " 38°. | 7.37 | | [7.34] | [7.57] | −0.03 | +0.20 |
| | *1924* | | | | | | | |
| " 26 | Dec. 12 | " " 38°. | | 7.18 | [7.27] | [7.52] | +0.09 | +0.34 |
| | " 17 | " " 38°. | | 7.41 | [7.50] | [7.73] | +0.09 | +0.32 |
| | " 22 | Serum as drawn. | | 7.38 | [7.51] | [7.74] | +0.13 | +0.36 |
| | *1926* | | | | | | | |
| | Mar. 23 | Separated serum 38°. | 7.26 | | [7.33] | [7.59] | +0.07 | +0.33 |
| | Apr. 16 | True serum 38°, 40 mm. | 7.30 | | [7.40] | [7.65] | +0.10 | +0.35 |
| " 30 | Feb. 11 | Separated serum 38°. | 7.30 | | [7.39] | | +0.09 | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Dog 32 | Apr. 1 | Separated plasma 38°. | 7.30 | | [7.32] | [7.54] | +0.02 | +0.24 |
| | " 3 | " " 38°. | 7.29 | 7.27 | [7.28] | [7.51] | +0.00 | +0.23 |
| " 33 | " 6 | " serum 38°. | 7.28 | | [7.33] | [7.59] | +0.05 | +0.32 |
| | " 22 | True serum 38°, 40 mm. | 7 39 | | [7.53] | [7.79] | +0.14 | +0.40 |
| | June 4 | " " 38°, 40 " | 7.27 | | [7.30] | [7.53] | +0.03 | +0.26 |
| | | " " 38°, 80 " | 7.08 | | [7.16] | [7.34] | +0.08 | +0.26 |

*Dog Serum or Plasma (Equilibrated at 20°).*

| Source. | Date. | Material.* | $pH_{20°}$ | | Indicator color. | | H correction. $[pH]_{20°} - pH_{20°}$ | C correction. $[pH]_{20°} - pH_{t°}$ |
|---|---|---|---|---|---|---|---|---|
| | | | Gasometric. | Electrometric. | $[pH]_{38°}$ | $[pH]_{20°}$ | | |
| | *1915* | | | | | | | |
| Dog 27 | Jan. 14 | Separated serum 20°. | 7.13 | | [7.08] | [7.23] | +0.10 | |
| | | | 7.90 | | [7.73] | [7.97] | +0.07 | |
| " 26 | Apr. 16 | True serum 20°, 40 mm. | 7.36 | | [7.23] | [7.45] | +0.09 | |
| " 33 | " 22 | " " 20°, 40 " | 7.43 | | [7.34] | [7.54] | +0.11 | |
| | June 4 | " " 20°, 40 " | 7.32 | | [7.19] | [7.38] | +0.06 | |
| | | " " 20°, 15 " | 7.60 | | [7.37] | [7.60] | +0.00 | |

*$CO_2$ tensions are only approximates.

TABLE I—*Continued.*

*Sheep Serum or Plasma (Equilibrated at 38°C. and 40 Mm. $P_{CO_2}$ except Where Otherwise Specified).*

| Source. | Date. | Material. | $pH_{38°}$ | | Indicator color. | | H correction. $[pH]_{38°} - pH_{38°}$ | C correction. $[pH]_{20°} - pH_{38°}$ |
|---|---|---|---|---|---|---|---|---|
| | | | Gasometric. | Electrometric. | $[pH]_{38°}$ | $[pH]_{20°}$ | | |
| | *1924* | | | | | | | |
| Sheep. | Dec. 10 | Serum as drawn. | | 7.17 | [7.20] | [7.40] | +0.03 | +0.23 |
| | *1925* | | | | | | | |
| | Jan. 8 | Separated serum 38°. | 7.33 | 7.32 | [7.29] | [7.49] | −0.03 | +0.17 |
| | | " " 38° to which $NaHCO_3$ was added. $CO_2$ = 160 mm. | 7.08 | | [7.08] | [7.14] | +0.00 | +0.06 |
| | | | 7.73 | | [7.71] | [7.86] | −0.02 | +0.13 |
| | " 20 | Separated serum. | 7.75 | | [7.74] | [7.95] | −0.01 | |
| | | | 7.15 | | [7.09] | [7.21] | −0.06 | +0.06 |
| | May 28 | True serum 38°, 40 mm. | 7.26 | | [7.26] | [7.46] | 0.00 | +0.20 |
| | | " " 38°, 80 " | 7.06 | | [7.04] | [7.21] | −0.02 | +0.15 |

(*Equilibrated at 20°.*)

| Source. | Date. | Material. | $pH_{20°}$ | | Indicator color. | | H correction. $[pH]_{20°} - pH_{20°}$ | C correction. $[pH]_{20°} - pH_{20°}$ |
|---|---|---|---|---|---|---|---|---|
| | | | Gasometric. | Electrometric. | $[pH]_{38°}$ | $[pH]_{20°}$ | | |
| | *1925* | | | | | | | |
| | Jan. 8 | Separated serum. | 7.57 | | [7.33] | [7.49] | −0.08 | |
| | May 28 | True serum 20°, 15 mm. | 7.60 | | [7.30] | [7.49] | −0.11 | |
| | | " " 20°, 40 " | 7.30 | | [7.04] | [7.21] | −0.09 | |

*Pooled Rat Serum (Equilibrated at 38°).*

| Source. | Date. | Material. | $pH_{38°}$ | | Indicator color. | | H correction. $[pH]_{38°} - pH_{38°}$ | C correction. $[pH]_{20°} - pH_{38°}$ |
|---|---|---|---|---|---|---|---|---|
| | | | Gasometric. | Electrometric. | $[pH]_{38°}$ | $[pH]_{20°}$ | | |
| | 1925 | | | | | | | |
| Rat. | Jan. 22 | Separated serum 40 mm. | 7.41 | 7.38 | [7.46] | [7.66] | +0.06 | +0.16 |
| | | " " 90 " | 7.19 | 7.16 | [7.20] | [7.36] | +0.02 | +0.18 |

*Human Patients. Serum or Plasma (Equilibrated at 38°C.).*

| Source. | Date. | Material. | Serum $pH_{38°}$ | | Indicator color. | | H correction. $[pH]_{38°} - pH_{38°}$ | C correction. $[pH]_{20°} - pH_{38°}$ |
|---|---|---|---|---|---|---|---|---|
| | | | Gasometric. | Electrometric. | $[pH]_{38°}$ | $[pH]_{20°}$ | | |
| | 1926 | | | | | | | |
| Human, nephritic patient. | Feb. 19 | Serum as drawn. | | 7.28 | [7.27] | [7.47] | −0.01 | +0.19 |
| Human, nephritic patient. | " 26 | Separated plasma equilibrated 33 mm., 38°. | 7.32 | | [7.34] | [7.58] | +0.02 | +0.26 |
| | Mar. 26 | Separated plasma equilitrated 39.7 mm., 38°. | 7.26 | 7.25 | [7.30] | [7.52] | +0.04 | +0.26 |
| Human patient, duodal ulcer. | " 3 | Separated serum equilibrated 38°, 40 mm. | 7.45 | | [7.55] | [7.80] | +0.10 | +0.35 |
| Human patient, hernia. | " 3 | Separated serum equilibrated 38°, 40 mm. | 7.43 | | [7.63] | [7.94] | +0.20 | +0.51 |

TABLE I—*Concluded.*

| Source. | Date. | Material. | Serum $pH_{38°}$ | | Indicator color. | | H correction. $[pH]_{38°} - pH_{38°}$ | C correction. $[pH]_{38°} - pH_{t_2°}$ |
|---|---|---|---|---|---|---|---|---|
| | | | Gasometric. | Electrometric. | $[pH]_{38°}$ | $[pH]_{20°}$ | | |
| | *1935* | | | | | | | |
| Human patient. | Mar. 6 | Separated serum equilibrated 38°, 40.3 mm. | 7.44 | | [7.62] | [7.93] | +0.18 | +0.49 |
| " " | " 7 | Separated serum 40 mm., 38°. | 7.44 | | [7.52] | [7.76] | +0.08 | +0.32 |
| " " encephalitic tachypnea. | " 20 | " " | 7.48 | | [7.46] | [7.72] | −0.02 | +0.24 |
| Human patients. | Feb. 11 | " sera 40 mm., 38°. | | | | | | |
| Carcinoma. | | | 7.43 | | [7.49] | | +0.06 | |
| Jaundice. | | | 7.46 | | [7.56] | | +0.10 | |
| Asthma. | | | 7.57 | | [7.67] | | +0.10 | |

TABLE II.

| Date. | Subject. | As drawn. | | | | | | Equilibrated at 38°C. | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $[CO_2]$ mM | | $[pH]_{38°}$ | | $[pH]_{24°}$ | | Material. | $[CO_2]$ mM | Gasometric pH. | $[pH]_{38°}$ | $[pH]_{20°}$ |
| | | Plasma. | Serum. | Plasma. | Serum. | Plasma. | Serum. | | | | | |
| *1925* | | | | | | | | | | | | |
| Mar. 23 | Dog 26 | 19.81 | 19.72 | [7.61] | [7.65] | [7.34] | [7.39] | Serum. | 19.87 | 7.26 | [7.33] | [7.59] |
| Feb. 18 | | 20.45 | 20.17 | [7.64] | [7.65] | [7.40] | [7.41] | — | — | — | — | — |
| Apr. 1 | " 32 | 20.38 | 20.35 | [7.66] | [7.64] | [7.41] | [7.39] | Plasma. | 21.17 | 7.29 | [7.32] | [7.54] |
| " 3 | | 20.15 | 20.00 | [7.67] | [7.68] | [7.42] | [7.42] | " | 20.85 | 7.29 | [7.29] | [7.51] |
| " 14 | " 27 | 23.71 | 23.88 | [7.75] | [7.75] | [7.50] | [7.49] | " | 25.01 | 7.37 | [7.34] | [7.56] |
| Mar. 18 | | 20.34 | 20.33 | [7.61] | [7.65] | [7.38] | [7.41] | Serum. | — | 7.31 | [7.40] | [7.60] |
| " 20 | Human patient (encephalitic tachypnea). | 27.50 | 27.58 | [7.73] | [7.78] | [7.48] | [7.53] | " | 32.11 | 7.48 | [7.46] | [7.72] |

The same variability is seen in the C correction ($[pH]_{20°} - pH_{38°}$) which enters into the determination of pH by Cullen's colorimetric method. It will be noted that the difference between the two colorimetric readings of the diluted serum, namely at 20° and at 38°, is much more constant than are the H and C corrections. This difference ($[pH]_{20°} - [pH]_{38°}$) will be discussed in a later paper where it will be shown that the change in pH, ($pH_{20°} - pH_{38°}$), of the diluted serum with temperature when the total $[CO_2]$ and available base are kept constant can be calculated. The color difference ($[pH]_{20°} - [pH]_{38°}$) is in our experience closely related to but not identical with the change in pH, ($pH_{20°} - pH_{38°}$) of the diluted serum.

The effect of change in pH upon the C and H corrections in a given serum we have not adequately studied but there is evidence in Table I, Dog 27 (Jan. 14, 1925)$_{38°}$, Dog 33 (June 4, 1925)$_{38°}$, and Dog 33 (June 4, 1925)$_{20°}$, that at least in the dog a considerable variation in these corrections may be found when there is a variation of 0.2 to 0.5 in pH.

The possibility that the H and C corrections might differ in serum and in plasma and might perhaps be more constant in one or the other has been tested in a series of instances presented in Table II. Blood was drawn in these experiments simultaneously through a Y tube connection into an oxalated and an unoxalated receiver. Colorimetric [pH] and $[CO_2]$ of both samples were determined on the serum and plasma as drawn. In most cases either serum or plasma was equilibrated and the gasometric pH and colorimetric [pH] of the equilibrated sample were determined.

The results show ($[pH]_{serum} - [pH]_{plasma}$) = + 0.05 to − 0.02. The difference between colorimetric reading of serum and plasma is therefore small compared with the possible magnitude of the H correction or with the possible variations in either H or C corrections.

*Effect of Dilution of Serum on the Colorimetric Reading of Serum.*

The use of a 1:20 dilution of serum in saline in Cullen's method is based on the conclusion of Cullen that this dilution is sufficient to render constant the protein effect on the color of the indicator. Cullen found that practically no further color change occurred

between dilutions from 1:20 to 1:40, his maximum dilution, and hence selected a 1:20 dilution as optimal.

We have subjected this question to further investigation with the hope originally that the use of greater dilutions with colorimetric readings at 38° would more or less completely obviate the H correction. In this aspect we were disappointed but the experiment brings out in a striking fashion the existence of a protein-salt-indicator effect over a wide range of dilution as well as the marked influence of the type of salt used as a diluent.

Dog serum was equilibrated at 38°C., the pH being determined gasometrically. Varying dilutions of the serum were made from 1:20 to 1:120 and the colors read at 38° against the Hastings bicolor standards at 38°C. Special precautions were taken in the preparation of the salt (NaCl and NaCNS, 0.150 M) solutions used as diluents. Water distilled from $H_2SO_4$ and $K_2Cr_2O_7$ and twice from $Ba(OH)_2$ and protected from the $CO_2$ of the air was used in preparing the saline. In addition, before being used, $CO_2$-free air was drawn through the saline for 1 to 2 hours and the necessary transfers made completely out of contact with air. These salt solutions showed by titration with 1/1000 N NaOH (made with the same water out of contact with laboratory air from concentrated NaOH) the following buffer value: 0.00186 mM NaOH per pH change of 7.2 to 7.8 for 100 cc. or 0.021 mM per 1 pH per liter or about 1/200 to 1/300 of the buffer value of the serum. The effect of the saline on the pH of the diluted serum was further controlled by making triplicate readings at all dilutions with saline adjusted with the N/1000 alkali to pH 6.8, 7.2, and 7.6, the undiluted serum being at pH 7.23.

The data are presented in Fig. 2, and show that a large H correction exists over a wide range of dilution having a maximum, with NaCl as diluent, of 0.14 at 1:20 but not disappearing even at a dilution of 1 to 120 where it is 0.08. When extrapolated to infinite dilution the colorimetric readings with NaCl diluent give approximately the gasometric pH of 7.23, but such a method of extrapolation from two or more points could hardly serve as a routine method, as the possibility of introducing error at high dilutions would be great. The shape of the curve with NaCl as diluent supports Cullen's choice of 1:20 as the optimum dilution.

The variations of the color of the diluted serum are not due to the reaction of the diluent since the readings are in close agreement in salines adjusted 0.4 pH units to the acid and alkaline side of the reaction of the serum. Using NaCNS at pH 7.2 the same general changes with dilution are apparent but the value of the H correction is much greater throughout.

In view of the observations presented in this paper we have come to the conclusion that for use with pathological sera, or under changing experimental conditions, the C or the H correc-

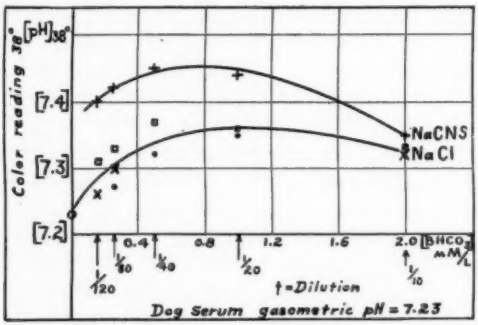


FIG. 2. Showing effect of dilution of a dog serum with NaCl and with NaCNS solutions upon phenol red indicator color at 38°; the true $pH_{38°}$ of the serum undiluted was 7.23.

\+ diluted with 0.150 M NaCNS solution, $pH_{38°} = 7.2$.
□ " " 0.150 M NaCl " $pH_{38°} = 7.6$.
× " " 0.150 M NaCl " $pH_{38°} = 7.2$.
● " " 0.150 M NaCl " $pH_{38°} = 6.8$.

tion must be determined on each serum if colorimetric readings made at 20° or at 38° respectively are to be relied upon. For this purpose we have adopted the following modification of the colorimetric method as described by Cullen at 20° or by Hastings and Sendroy at 38°.

1. The indicator color [pH], of a sample of serum (or plasma) is read at 20° or at 38° by the method of Cullen or of Hastings and Sendroy.

2. 5 cc. of the serum (or plasma) is equilibrated in a tonometer of 150 to 200 cc. volume at 38° with $p_{CO_2}$ selected to give a pH

near the pH as drawn, the equilibration being done by the second method described by Austin, Cullen, Hastings, McLean, Peters, and Van Slyke (1922), performing only one equilibration and correcting the tonometer $p_{CO_2}$ by analysis of the $[CO_2]$ of the serum (or plasma) before and after equilibration.

3. The equilibrated serum (or plasma) is analyzed by Van Slyke's method for its $[CO_2]$, and its colorimetric [pH] determined in the same way and at the same temperature as in step 1.

4. The $pH_{38°}$ of the equilibrated serum (or plasma) is calculated by the Henderson-Hasselbalch equation using Bohr's solubility coefficient for $CO_2$ in serum (0.975 $\alpha$ water) and $pK' = 6.10$, or Van Slyke, Wu, and McLean's (1923) solubility coefficient (0.914 $\alpha$ water) and $pK' = 6.07$ in accordance with the data of Cullen, Keeler, and Robinson (1925).

5. The value $([pH]_{20°} - pH_{38°})$ or $([pH]_{38°} - pH_{38°})$ is determined from the data of steps 3 and 4 on the equilibrated serum (or plasma) and the same C or H correction applied to the colorimetric reading of the serum (or plasma) as drawn in step 1.

This procedure has checked within $\pm 0.03$ pH the electrometric determinations of the $pH_{38°}$ of the undiluted serum or plasma as drawn in every instance where we have made the comparison.

## CONCLUSION.

It is shown that under pathological conditions in man and in the dog under normal and experimental conditions there is considerable *variation in the difference* between colorimetric reading of the [pH] of diluted serum or plasma either at 20° or at 38° and the true pH of the undiluted serum or plasma at 38°.

A method of determining ([pH] − pH) for making this correction is outlined.

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# A STUDY OF THE RETENTION OF URIC ACID DURING FASTING.*

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In a previous paper (1) we have reported that during prolonged fasting in the adult human subject there is a remarkable increase in the concentration of uric acid in the blood. Each of the twenty-eight fasting periods studied showed an increase. In the twenty-one periods which lasted 8 days or longer, the average increase in blood uric acid was 165 per cent; *i.e.*, from an average prefasting level of 4 mg., the concentration rose to a fasting peak of 10.6 mg. per 100 cc. of whole blood. The highest values encountered were 16 mg. per 100 cc. of whole blood, and 22.5 mg. per 100 cc. of plasma. Hoeffel and Moriarty (2) have found that an increase occurs in fasting children. Morgulis and Edwards (3) have reported the same for fasting dogs.

In the previous paper it was stated simply that the increase of uric acid in the blood was the result of decreased uric acid elimination. This paper deals with (1) the evidence for this conclusion, (2) the quantitative relationship between uric acid in urine, blood, and tissues, and (3) various food and other factors which modify the retention.

## *Material and Methods.*

Observations have been made during the past 3 years. Two of the subjects were healthy male adults. The other twenty-two

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were patients who were undergoing fasting as treatment for recurring convulsions. Their ages ranged from 15 to 38 years. None had any evidence (which included the usual laboratory tests) of kidney lesion. Fasting (except for the healthy subject W. L. who fasted in the laboratory) was conducted on the open wards of the hospital. Unless otherwise stated, water was taken *ad libitum*. Urine and blood collections were made by the writer. There was no material difference, with respect to uric acid retention, in the results obtained from patients and from the healthy controls.

The following analytical methods were used: blood uric acid, Folin (4) as a routine, with occasional duplicate determinations using (1) preliminary precipitation with silver lactate (employed as a routine with subject L. R., Table XIX), (2) the method of Folin-Wu (5), and (3) the method and reagent of Benedict (6). A comparison of results by these methods has been given elsewhere (7). One must be cautious in accepting high measurements of uric acid in the blood because of the uncertainty concerning the specificity of present methods of analysis. Certain substances in the blood react with the uric acid reagent to give the same blue color that uric acid gives. Benedict (8) says that this unknown substance is precipitated by silver lactate. On the other hand there is no such doubt concerning the accuracy of present methods of measuring uric acid in urine. On account of the fact that during fasting decrease of uric acid in urine is greater than the simultaneous increase in blood, it would seem that values found for the blood are not too high. Other methods used were: nonprotein nitrogen, Folin-Wu (5); blood amino acid nitrogen, Folin (9); plasma bicarbonate, Van Slyke (10); urine uric acid, Folin-Wu (11); urine total nitrogen, Folin-Denis (12); urine pH, colorimetrically as for blood serum, Cullen (13), except that it was not found necessary to collect urine under oil.

### *The Evidence for Retention.*

If we had only the evidence of the increased concentration of uric acid in the blood, we should need to consider the possibility of either decreased destruction of uric acid or increased production (as from increased breakdown of body protein). The latter possibility is rendered improbable because neither nitrogen excretion

TABLE I.

*Uric Acid Concentration in Blood and Urine during 14 Days of Fasting.*

Healthy subject. W. L., male, age 38, weight 64.3 kilos (at beginning of fast). Measured fluid intake during fast: 900 cc. distilled water first 2 days, 1200 cc. on subsequent days.

| Diet. | Day. | Blood. | | | | Urine. | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Per 100 cc. | | | | | Uric acid. | | |
| | | Uric acid. | | Concentration of uric acid of urine / blood. | Plasma bicarbonate. | Amount. | Per 100 cc. | 24 hr. output. | Total N. |
| | | Whole blood. | Plasma. | | | | | | |
| | | *mg.* | *mg.* | | *vol. per cent $CO_2$* | *cc.* | *mg.* | *mg.* | *gm.* |
| Purine-free. | 1 | 4.55 | | 12.1 | 70. | 785 | 55 | 428 | 7.74 |
| | 2 | | | | | 856 | 50 | 429 | 7.69 |
| " | 3 | 3.48 | | 15.5 | | 820 | 54 | 445 | 7.81 |
| " | 4 | 3.19 | 4.17 | 16.3 | | 743 | 52 | 386 | 7.32 |
| Fast. | 1 | 3.56 | 3.81 | 23.9 | 69.7 | 488 | 85 | 416 | 6.67 |
| " | 2 | 5.15 | 5.48 | 5.4 | 62.2 | 615 | 28 | 173 | 8.97 |
| " | 3 | 6.15 | 9.30 | 1.1 | 48.6 | 1355 | 7 | 101 | 12.19 |
| " | 4 | 8.99 | 11.27 | 1.7 | 40.5 | 1600 | 15 | 249 | 11.58 |
| " | 5 | 10.31 | 14.71 | 2.6 | 40.2 | 1020 | 27 | 282 | 8.54 |
| | | 9.25 | | | 33.2 | | | | |
| " | 6 | 11.05 | 13.68 | 3.4 | 36.4 | 745 | 37 | 274 | 7.34 |
| | | 10.54 | | | | | | | |
| " | 7 | 12.35 | 14.81 | 3.6 | 45.1 | 670 | 45 | 301 | 6.99 |
| | | 12.35 | | | | | | | |
| " | 8 | 11.30 | | 2.8 | 44.7 | 870 | 32 | 276 | 7.54 |
| | | | | | 46.7 | | | | |
| " | 9 | 10.10 | 13.80 | 3.2 | 52.1 | 740 | 32 | 235 | 6.31 |
| " | 10 | 10.30 | 11.34 | 3.3 | 49.6 | 760 | 34 | 259 | 6.82 |
| " | 11 | 10.40 | 13.50 | 2.7 | 54.0 | 905 | 28 | 255 | 7.13 |
| " | 12 | 10.69 | 10.59 | 2.6 | 54.0 | 1120 | 28 | 315 | 6.66 |
| | | 10.05 | | | | | | | |
| " | 13 | 9.39 | | 4.6 | 52.3 | 690 | 43 | 300 | 5.87 |
| " | 14 | 9.13 | 11.84 | 4.3 | 51.0 | 885 | 40 | 350 | 6.38 |
| | | 10.31 | | | | | | | |

TABLE I—*Concluded.*

| Diet. | Day. | Blood. Per 100 cc. Uric acid. Whole blood. | Blood. Per 100 cc. Uric acid. Plasma. | Blood. Concentration of uric acid of $\frac{\text{urine}}{\text{blood}}$ | Blood. Plasma bicarbonate. | Urine. Amount. | Urine. Uric acid. Per 100 cc. | Urine. Uric acid. 24 hr. output. | Urine. Total N. |
|---|---|---|---|---|---|---|---|---|---|
| | | *mg.* | *mg.* | | *vol. per cent $CO_2$* | *cc.* | *mg.* | *mg.* | *gm.* |
| Cream.* | | 11.50<br>10.75 | 12.90 | 4.9 | 53.9<br>49.3 | 735 | 57 | 421 | 6.53 |
| Low protein, purine-free.† | 1 | 6.35<br>3.99 | 7.61 | 15.0 | 66.0<br>71.0 | 975 | 97 | 952 | 4.47 |
| | 2 | 3.81 | 5.00 | 32.0 | 72.5 | 515 | 122 | 630 | 3.73 |
| | 3 | 3.53<br>3.34 | 4.57 | 40.6 | 73.2 | 315 | 142 | 449 | 3.25 |
| | 4 | 3.22 | 3.81 | 65.5 | 72.5 | 305 | 211 | 649 | 3.90 |
| | 5 | 2.70 | 2.74 | 27.4 | 74.4 | 955 | 74 | 710 | 5.18 |
| High protein, purine-free.‡ | 1 | 2.44<br>2.91 | 2.91 | 19.6 | 77.8<br>71.3 | 750 | 47 | 356 | 6.59 |
| | 2 | 2.00<br>2.00 | 2.19 | 32.0 | 75.0 | 770 | 64 | 492 | 9.59 |
| | 3 | 2.22<br>2.25 | 2.50 | 27.4 | 68.2 | 780 | 63 | 495 | 13.66 |
| Regular, containing purine. | 1 | 2.79 | 2.82 | 24.3 | 66.3 | 660 | 68 | 449 | 9.59 |
| | 2 | 3.15 | 3.27 | 25.1 | 67.5 | 660 | 78 | 512 | 6.40 |
| | 3 | 4.00 | 4.37 | 15.0 | 66.2 | 1020 | 60 | 611 | 5.33 |
| | 4 | 4.04 | 4.32 | 15.0 | 68.2 | 1245 | 60 | 746 | 5.88 |

* 500 cc. 40 per cent cream representing 200 gm. fat, calories 1860.

† Average of 5 days. Protein 28 gm., fat 166 gm., carbohydrate 299 gm., calories 2882.

‡ Average of 3 days. Protein 155 gm., fat 307 gm., carbohydrate 375 gm., calories 5126.

In this table blood for each day was drawn near the middle of the 24 hour urine period for that day. Second samples were taken 5 to 6 hours later.

nor non-protein nitrogen of the blood paralleled the concentration of uric acid in the blood. Both possibilities are ruled out by the fact that the increased concentration in the blood is more than accounted for by the decreased excretion of uric acid in the urine.

The uric acid output was measured during twenty-one of the fasting periods, and in every case a decrease in the uric acid excretion was found. Data concerning the 14 day fast of the healthy

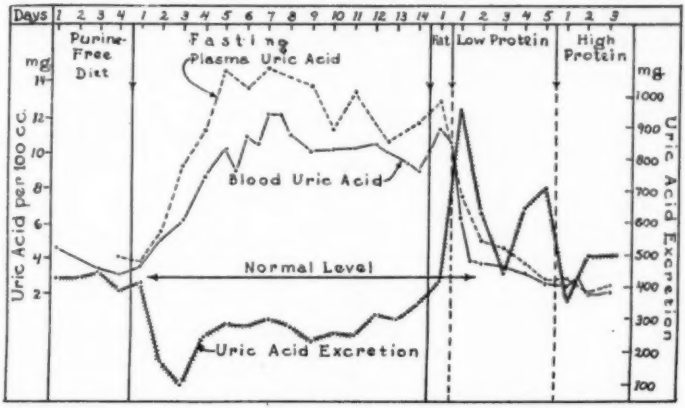


FIG. 1. (Illustrating part of Table I.) 14 day fast of healthy subject W. L. showing increase of uric acid in blood, in plasma, and decreased uric acid excretion. In this and subsequent similar figures, equal scale divisions on the ordinate represent equal amounts of uric acid in blood and urine, based on the calculated blood volume of the subject at the beginning of fasting. For example, W. L. had a calculated blood volume of 5.9 liters. A fall of 1 mg. of uric acid per 100 cc. of blood, if excreted, would increase uric acid output for the period by 59 mg. Therefore, 1 mg. for blood concentration and 59 mg. for uric acid excretion have equal values on the scale.

subject W. L. are presented in Table I. (In this table, blood was drawn each day near the middle of the 24 hour collection of urine recorded for that day.) Table I is illustrated in part by Fig. 1. (In this and other figures, equal scale divisions on the ordinate represent equal amounts of uric acid in blood and urine, taking into account the calculated blood volume of the subject. For example, W. L. weighed 64.3 kilos and had an approximate blood

volume of 5.9 liters. A fall of 1 mg. of uric acid per 100 cc. of blood, if all were excreted, would result in an increase of 59 mg.

TABLE II.

*The Uric Acid of Blood and Urine during 21 Days of Fasting.*

Epileptic patient. S. S., female, age 19, weight 58 kilos.

| Diet. | Day. | Blood.* Uric acid per 100 cc. | Urine. | | | | Concentration of uric acid of $\frac{\text{urine}}{\text{blood}}$ |
|---|---|---|---|---|---|---|---|
| | | | Volume 24 hrs. | Uric acid. | | Total N. | |
| | | | | Per 100 cc. | Total 24 hr. output. | | |
| | | *mg.* | *cc.* | *mg.* | *mg.* | *gm.* | |
| Fast. | 1 | 4.0 | 950 | 44 | 418 | 2.7 | 11.0 |
| " | 2 | 5.8 | 930 | 35 | 323 | 4.2 | 5.9 |
| " | 3 | | 1730 | 22 | 388 | | |
| " | 4 | 7.6 | 1400 | 22 | 314 | | 2.9 |
| " | 5 | 7.7 | 650 | 27 | 186 | 6.2 | 3.5 |
| " | 6 | | 950 | 19 | 184 | | |
| " | 7 | 8.4 | 1120 | 12 | 139 | 5.6 | 1.5 |
| " | 8 | | 1060 | 16 | 167 | | |
| " | 9 | | 1470 | 12 | 175 | | |
| " | 10 | 9.8 | 1000 | 15 | 147 | 5.1 | 1.5 |
| " | 11 | 13.5 | 950 | 18 | 169 | | 1.3 |
| " | 12 | 9.5 | 1030 | 38 | 392 | | 4.0 |
| " | 13 | 10.9 | 1200 | 26 | 318 | | 2.4 |
| " | 14 | 10.3 | 960 | 29 | 278 | 4.1 | 2.8 |
| " | 15 | 10.3 | 950 | 52 | 494 | | 5.0 |
| " | 16 | 9.8 | 1040 | 37 | 386 | 4.6 | 3.8 |
| " | 17 | 9.5 | 1270 | 43 | 553 | | 4.6 |
| " | 18 | 11.2 | 920 | 43 | 394 | 3.5 | 3.8 |
| " | 19 | 11.5 | 960 | 27 | 267 | | 2.3 |
| " | 20 | 11.2 | 920 | 28 | 250 | 4.1 | 2.5 |
| " | 21 | 11.3 | 960 | 37 | 353 | | 3.2 |
| Purine-free. | 1 | 9.9 | 740 | 57 | 422 | 4.9 | 12.1 |
| " | 2 | 4.7 | 380 | 89 | 337 | | 24.0 |
| " | 3 | 3.7 | 1010 | 67 | 676 | 6.7 | 20.3 |
| " | 4 | 3.3 | 1000 | 91 | 910 | 6.6 | 33.7 |

* In this and subsequent similar tables, blood for each day was drawn near the beginning of the 24 hr. urine collection recorded for that day.

in the urine. Therefore in Fig. 1, 1 mg. for blood and 59 mg. for urine have equal values on the scale.)

Inspection of Fig. 1 shows a marked drop in uric acid excretion

during the first few days of fasting with coincident but less marked increase in the concentration of uric acid in the blood. The maximum concentration in the blood (12.3 mg.) was reached on the 7th day. At this point an apparent equilibrium was established and uric acid remained high in blood and low in urine until the end. Immediately on breaking the fast by means of a purine-free, low protein diet there was a precipitous drop in the uric acid of the blood (a fall of nearly 7 mg. per 100 cc. in 12 hours) with coincidently large excretion of uric acid (678 mg. in 12 hours). The quantitative relationships between uric acid in blood and urine will be discussed later (Table V, Fig. 6).

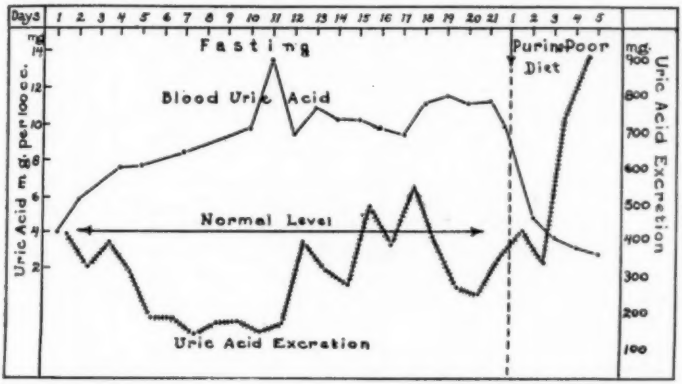


FIG. 2. (Illustrating a part of Table II.) 21 day fast of epileptic subject S. S. showing increase of blood uric acid, and decrease of uric acid excretion throughout fasting. Note the great increase in uric acid excretion without decrease of uric acid in the blood on the last day of observation.

The maximum retention obtained varied with different subjects. The lowest 24 hour output of uric acid encountered was 78 mg. (Table XIX). The lowest 24 hour output of the subject showing the highest level of excretion was 324 mg. (Table VIII). The lowest 24 hour output of all subjects averaged 161 mg.

The question arises, for how long would this retention of uric acid be maintained? The longest starvation period in this series was 21 days. Data concerning it are presented in Table II and Fig. 2. (In this and subsequent tables, blood was drawn each

day near the beginning of the 24 hour urine collection for that day.) Though there were fluctuations during the 3rd week of the fast, the abnormal reciprocal levels were maintained, or nearly maintained, till the end.

TABLE III.

*Diurnal Variation in Excretion of Uric Acid and Nitrogen during Fasting.*

Healthy subject. W. L., age 38, weight 64.3 kilos.

Water intake during fasting 500 cc. at night and 700 cc. during day.

| Diet. | Day. | Uric acid excretion. | | Total N. | |
|---|---|---|---|---|---|
| | | Night. 8 p.m.—8 a.m. | Day. 8 a.m.—8 p.m. | Night. 8 p.m.—8 a.m. | Day. 8 a.m.—8 p.m. |
| | | *mg.* | *mg.* | *gm.* | *gm.* |
| Purine-free. | 1 | 178 | 250 | 3.38 | 4.36 |
| " | 2 | 148 | 281 | 3.72 | 3.97 |
| " | 3 | 176 | 269 | 3.49 | 4.32 |
| " | 4 | 158 | 228 | 3.66 | 3.66 |
| Fast. | 1 | 176 | 240 | 3.08 | 3.59 |
| " | 2 | 103 | 71 | 3.84 | 5.13 |
| " | 3 | 46 | 55 | 6.74 | 5.45 |
| " | 4 | 69 | 180 | 6.38 | 5.20 |
| " | 5 | 171 | 111 | 4.28 | 4.26 |
| " | 6 | 150 | 124 | 4.08 | 3.27 |
| " | 7 | 156 | 145 | 3.55 | 3.44 |
| " | 8 | 144 | 132 | 3.50 | 4.04 |
| " | 9 | 106 | 129 | 2.76 | 3.55 |
| " | 10 | 121 | 138 | 3.35 | 3.47 |
| " | 11 | 134 | 121 | 3.43 | 3.70 |
| " | 12 | 174 | 141 | 3.43 | 3.23 |
| " | 13 | 154 | 146 | 2.98 | 2.89 |
| " | 14 | 159 | 191 | 3.22 | 3.16 |
| Fat. | 1 | 181 | 240 | 2.95 | 3.58 |
| Low protein, purine-free. | 1 | 678 | 274 | 3.06 | 1.41 |
| " | 2 | 378 | 252 | 2.00 | 1.73 |
| " | 3 | 252 | 197 | 1.84 | 1.41 |
| " | 4 | 201 | 448 | 2.49 | 1.41 |
| " | 5 | 477 | 233 | 3.01 | 2.17 |
| High protein, purine-free. | 1 | 179 | 177 | 3.11 | 3.48 |
| " | 2 | 201 | 291 | 5.11 | 4.48 |
| " | 3 | 252 | 243 | 7.82 | 5.84 |

*Diurnal and 4 Hourly Variations in Excretion.*

On a constant, purine-free diet, as is well known, the elimination of uric acid during the day is greater than during the night. During fasting this diurnal inequality of excretion disappeared. Table

TABLE IV.

*Observations on Uric Acid Retention at 4 Hourly Intervals.*

Healthy subject. W. L., age 38, weight 68 kilos.

| Blood. | | | | Urine. | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Per 100 cc. | | | | | Uric acid. | | | |
| Hr. taken. | Uric acid. | Non-protein N. | Amino acid N. | Period. | Volume. | Per 100 cc. | 24 hr. output. | Total N. | pH |
| | *mg.* | *mg.* | *mg.* | | *cc.* | *mg.* | *mg.* | *gm.* | |
| Apr. 17* | | | | Apr. 17 | | | | | |
| 11.00 p.m. | 3.52 | 28.6 | | 8–12 p.m. | 55 | 68 | 37 | 0.887 | 5.6 |
| Apr. 18 | | | | Apr. 18 | | | | | |
| 3.00 a.m. | 3.43 | 31.6 | 5.55 | 12–4 a.m. | 142 | 36 | 50 | 1.18 | 5.8 |
| 6.30 " | 3.73 | 28.3 | 5.05 | 4–8 " | 330 | 22 | 71 | 1.47 | 5.9 |
| 10.00 " | 3.67 | 30.3 | 5.11 | 8–12 " | 205 | 38 | 78 | 1.46 | 5.9 |
| 2.30 p.m. | 4.00 | 30.0 | 5.07 | 12–4 p.m. | 105 | 37 | 39 | 1.10 | 5.6 |
| 6.30 " | 4.15 | 31.9 | 5.64 | 4–8 " | 70 | 39 | 27 | 1.09 | 5.4 |
| 10.00 " | 4.65 | 36.8 | 5.30 | 8–12 " | 90 | 23 | 20 | 1.07 | 5.4 |
| Apr. 19 | | | | Apr. 19 | | | | | |
| 2.00 a.m. | 4.55 | 35.3 | 5.50 | 12–4 a.m. | 120 | 21 | 25 | 1.46 | 5.3 |
| 6.00 " | 5.26 | 39.5 | 5.10 | 4–8 " | 168 | 10 | 17 | 1.68 | 5.2 |
| 11.00 " | 5.15 | 40.3 | 5.28 | 8–12 " | 170 | 11 | 19 | 1.62 | 5.2 |
| 2.00 p.m. | 5.33 | 41.2 | 5.60 | 12–4 p.m. | 492 | 4 | 18 | 2.49 | 5.0 |
| 6.00 " | 6.38 | 40.0 | 5.00 | 4–8 " | 165 | 11 | 18 | 1.56 | 5.2 |
| 12 hr. interval. Purine-free food at 8 p.m., Apr. 19, and 8 a.m., Apr. 20. | | | | | | | | | |
| Apr. 20 | | | | Apr. 20 | | | | | |
| 10.30 a.m. | 3.90 | 43.5 | 5.83 | 8–12 a.m. | 70 | 59 | 41 | 1.30 | 5.8 |

* Last food 2 p.m., Apr. 17.

III (presenting further data concerning the 14 day fast of W. L.), demonstrates this.

Normally, there is also a fairly constant hourly variation in excretion. Observations made every 4 hours showed that the periodic variation also disappeared (Table IV and Fig. 3. See

also Figs. 8 and 12). The same was true for observations made every hour. During fasting, excretion from hour to hour, as well as from day to day, is maintained at a relatively low flat level.

*Quantitative Relationships between Uric Acid of Tissues, Blood, and Urine.*

Is the amount of uric acid in the body a fixed quantity? This question is of fundamental importance in the interpretation of

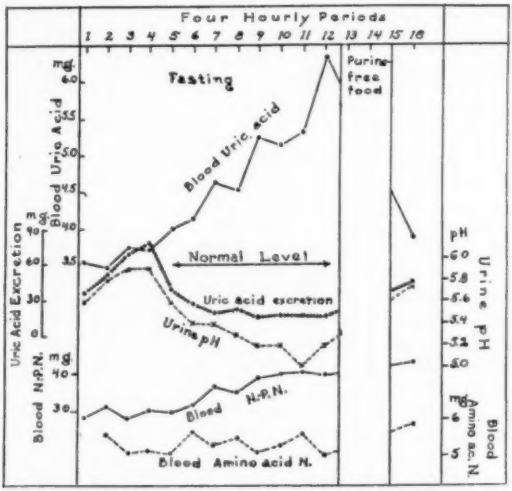


FIG. 3. (Illustrating a part of Table IV.) 54 hour fast of healthy subject W. L. with observations every 4 hours, showing disappearance of uric acid tide after 24 hours of fasting, and its apparent relation to urinary pH. The blood uric acid (solid line) shows no constant relation to blood non-protein nitrogen or amino acid nitrogen.

observed variations in the elimination of uric acid. Most workers who have studied the effect of various foods in normal subjects have regarded the amount as fixed, or, in other words, the renal threshold as constant, and have interpreted variations in uric acid elimination as evidence of variations in the production or destruction of uric acid in the body. The evidence which will be presented in the paper proves, we believe, that this conception is erroneous.

Before attempting a statement of the quantitative relationships of uric acid in tissues, blood, and urine, it is necessary to have in mind the approximate volumes of water in these three substances. Fig. 4 represents the relative amounts of water in an individual weighing 70 kilos. Expressing the relationship in round numbers, the body water (outside that in blood) is 30, blood 4, and urine 1. Since the volume of body water outside the blood is approximately seven times the volume of blood, it is evident that a decrease of 1 mg. of uric acid in 100 cc. of tissue water, if it all entered the blood, would increase the concentration in the blood by approximately 7 mg. per 100 cc. If all the uric acid went into the urine, it would increase the uric acid output by about 470 mg. If this

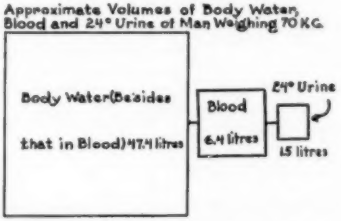
Approximate Volumes of Body Water, Blood and 24° Urine of Man Weighing 70 K.G.
Body Water(Besides that in Blood) 47.4 litres
Blood 6.4 litres
24° Urine 1.5 litres

FIG. 4. Approximate volumes of body water (besides that in blood), of blood, and of 24 hour urine of a subject weighing 70 kilos. Calculation is based on assumption that body water in liters is 75 per cent, and blood in liters is 9.2 per cent of body weight in kilos; and that blood is 80 per cent water. (The last named correction is not used in calculations elsewhere in this paper.)

transfer took place in 24 hours, the uric acid elimination for that day would be increased about 100 per cent. If the transfer occupied only an hour, the period of time used by some investigators, the elimination would be increased about 2400 per cent.

Fig. 5 shows the approximate relative amounts of uric acid in the body water (besides that in blood), blood, and 24 hour urine, of an individual weighing 70 kilos. The computation is based on the assumption that the 24 hour elimination is 7 mg. per kilo, that the concentration of uric acid in blood is 4 mg. per 100 cc., and that the concentration in body water, aside from blood, is 2 mg. per 100 cc. This last assumption contains considerable chance for error. Most of the weight of the body is in its muscle.

100 gm. of human muscle contain approximately 2 mg. of uric acid (14). Muscle water would contain more, about 2.7 mg. per 100 cc. It would seem, therefore, that 2 mg. is not too high an estimate for the average concentration of uric acid in body water lying outside the blood. In round numbers the relative amounts of uric acid in these three reservoirs are body water (aside from that in blood) 4, blood 1, 24 hour urine 2.

From the point of view of quantitative relationships Figs. 1 and 2 are misleading, because the curve of blood uric acid represents the total accumulation in the blood, whereas the curve of uric acid excretion represents day to day values. Table V and Fig. 6 present the data from Table I (compressed into 3 day

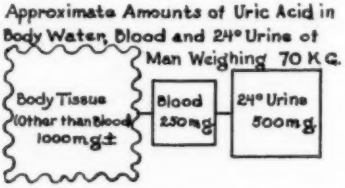


FIG. 5. Approximate amounts of uric acid in body water (besides that in blood), in blood, and in 24 hour urine, of a subject weighing 70 kilos. Calculation is based on the assumption that the average uric acid concentration per 100 cc. is 2 mg. in body water, and 4 mg. in blood and that the 24 hour endogenous output is 7 mg. per kilo of body weight. The wavy outline of the area representing body water indicates that the figure of 1000 mg. is only a rough approximation.

periods) with this discrepancy corrected. Table V and Fig. 6 show the accumulation of uric acid in blood and plasma in comparison with the total *decreased* elimination. The amount of decreased excretion of uric acid is based on the previously determined endogenous excretion. Inspection of Fig. 6 shows that in this fasting period, increase of uric acid in the blood was more than accounted for by decreased elimination in the urine. Most of the conclusions concerning quantitative relationships are based on the data obtained from the fasting periods of subject W. L. These data are especially valuable because the subject was healthy, analyses of blood and urine were begun soon after collection, and, since the writer was the subject, there was no question concerning either the completeness of the fast, or the accuracy of urine collections.

All of the fasting periods in which it was possible to get satisfactory measurements of endogenous excretion showed a total retention of uric acid greater than the accumulation in the blood.

TABLE V.

*Quantitative Relationship between Decreased Uric Acid Excretion and Increased Uric Acid Content of Blood during Fasting.**

Healthy subject, W. L. At beginning of fast, weight 64.3 kilos, estimated volume of blood 5.91 l., of plasma 3.05 l. At end of fast, weight 57 kilos, estimated volume of blood 5.24 l., plasma 2.73 l. Prefasting endogenous uric acid excretion 422 mg. a day.

| Day of experiment. | Diet. | Uric acid excretion. | | | Surplus in blood. | | Net decreased excretion.† |
|---|---|---|---|---|---|---|---|
| | | Expected. | Actual. | Decrease from expected. | Whole. | Plasma. | |
| | | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* |
| End of 3rd. | Fast. | 1266 | 584 | 682 | 322 | 231 | 360 |
| " " 6th. | " | 2532 | 1476 | 1056 | 483 | 319 | 573 |
| " " 9th. | " | 3798 | 2253 | 1545 | 358 | 211 | 1187 |
| " " 12th. | " | 5064 | 3115 | 1949 | 308 | 205 | 1640 |
| " " 14th. | Fast 1½ days. | 5908 | 3792 | 2116 | 413 | 245 | 1703 |
| " " 16th. | Fat ½ day. " ½ " | 6750 | 5362 | 1388 | 13 | 32 | 1375 |
| " " 20th. | Low protein 1½ days. " " 4 " | 8440 | 7601 | 839 | −61 | −21 | 900 |
| " " 23rd. | High " 3 " | 9706 | 8984 | 722 | −45 | −31 | 767 |

* The same fasting period as recorded in Table I. Urine was collected in 12 hr. periods. In this table, fasting was counted as beginning 12 hrs. after last food was eaten, instead of when it was eaten, as in Table I. In this table, therefore, blood was taken near the beginning and end of each 24 hr. urine period. In the calculations, approximate allowance is made for changes in blood volume during and after starvation. In the postfasting period, food was purine-free.

† *I.e.*, amount of decreased excretion not accounted for by increased uric acid content of whole bloood.

The retained uric acid not found in the blood, with certain possible losses which will be considered, presumably remained in the tissues. Following the taking of food most of this retained uric acid was excreted in the urine.

*Proportion of Retained Uric Acid Not Recovered.*

In the case of W. L., the total uric acid eliminated during 14 days of fasting, 1 day of cream, and 8 of mixed diet, was 767 mg. less than the calculated total endogenous excretion for these 23 days (last column of Table V).

There are three possible explanations for this uric acid loss, *viz.* delayed excretion, decreased production, and increased destruction.

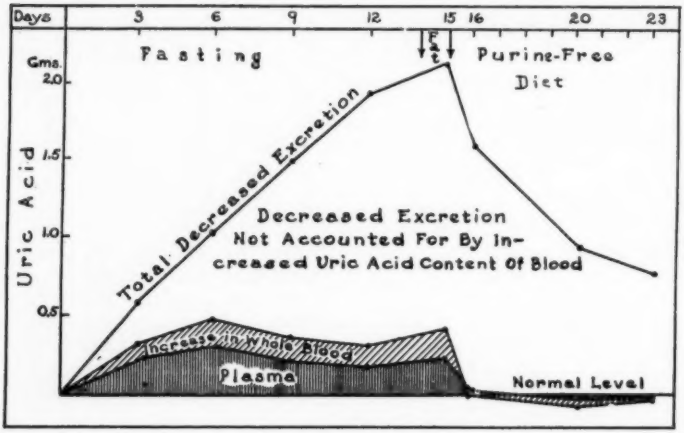


FIG. 6. (Illustrating Table V.) Total uric acid retention during and subsequent to the 14 day fast of healthy subject W. L. (see Fig. 1). The total retention of uric acid (the calculated normal endogenous excretion for the period minus the actual excretion) is indicated by the area bordered by outer solid line. The amount of this uric acid retained in blood is indicated for whole blood by the diagonally hatched area and for plasma by the perpendicularly hatched area. 64 per cent of the uric acid retained during fasting was excreted in the postfasting period.

1. All the uric acid which was retained in the tissues during fasting may not have been excreted even after 8 days of mixed diet. Folin, Berglund, and Derick (15) "have no faith in the concept of delayed excretion." In these fasting cases, however, postfasting uric acid excretion, even with constant, purine-free diet, was very irregular. On three occasions, there was a wave of increased uric acid excretion which came 4 to 7 days after the break-

ing of fast, and after uric acid excretion had touched a normal level (Figs. 2, 7, and 10). Unfortunately in each case this wave started on the day in which the subject left the hospital, and the extent of the increased output was not learned. The probability of retained uric acid is lessened in the case of the subject W. L., because he was on a high protein diet for 3 of the 8 postfasting days, which should have reduced stored uric acid to a minimum.

2. It is possible that coincident with the decrease of 11 per cent in weight and the lessened basal metabolic rate during fasting, decreased production of uric acid was sufficient to account for the 13 per cent deficit observed. On the other hand, the weight lost was mostly water loss; we know of no observations showing that uric acid production varies with metabolic rate, and protein metabolism was increased rather than decreased during fasting (*cf.* Total nitrogen column, Table I). There are, therefore, no definite grounds for thinking that decreased production was the cause of the uric acid loss.

3. Of the 1703 mg. which were retained at the end of 13½ days of fasting and 12 hours of cream diet, 64 per cent was recovered during the succeeding 8 days. In another 6 day fasting period of W. L. in 3 postfasting days there was a 63 per cent recovery (Table XV, Fig. 8). The smallest recovery was in the fast of S. S. (Table II). At the end of 21 days fasting, she had retained approximately 3 gm. of uric acid. Only about 20 per cent of this was found in the urine during 4 postfasting days. This subject was one of those who passed a large amount of uric acid on the last day of observation, so that the proportion recovered undoubtedly would have been greater if the period of observation had been longer. When uric acid is injected intravenously, the amount recovered in the 3 or 4 days which follow varies greatly, but the average is about 50 per cent. Folin, Berglund, and Derick (15), believe that the unrecovered portion is destroyed and that the destruction of uric acid in the body is more rapid when its concentration in the blood is high. During fasting, concentration of uric acid was maintained at a high level for a week or more, so that, if we accept the opinion of Folin and associates, a 64 per cent recovery is surprisingly large. Our data do not permit decision as to whether the unrecovered portion of uric acid was retained in the tissues or destroyed. It is possible, of course, that all three of the factors named played a part.

*Proportion of Retained Uric Acid Contained in Tissues.*

The data in Table V permit us to estimate the increased concentration of uric acid in the tissues at the end of fasting. At this time the total decrease from the expected amount of urinary uric acid was 2116 mg. Of this amount 245 mg. were in blood plasma, and 767 mg. were not recovered subsequently, and may have been destroyed. The balance, 1104 mg., must have been contained in the tissues, including cells of the blood. This amount would raise the concentration of uric acid in tissue water by approximately 2.76 mg. per 100 cc. Folin, Berglund, and Derick (15), estimate that after Berglund had received intravenous injection of 2 gm. of uric acid, the concentration of uric acid in his tissues was increased by 2.3 mg. per 100 gm.

As a result of extensive uric acid injection experiments these authors state:

". . . . it is quite impossible to apply to uric acid the concept of free and prompt distribution within the animal body and this absence of distribution cannot very well be explained except on the basis of impermeability on the part of living tissues. The resistance of living tissues to uric acid can be overcome to a certain extent by means of a high and sustained uric acid pressure. . . . . Sustained pressure of abnormally high uric acid levels in the blood does, therefore, seem to force the muscles to take up uric acid, up to concentrations equal to from 8 to 15 per cent of that in the blood."

On account of the fact that during fasting uric acid concentration in blood requires days in which to reach its peak, fasting offers opportunity for measuring the distribution of uric acid at various rising levels of concentration in the blood. Table VI shows the sequence of events in the case of W. L. First, although the retained uric acid originated in the tissues, the retention showed itself first in the blood. During the first 24 hours of starvation 63 per cent of the retained uric acid was found in plasma. This is evidence of an elevated renal threshold, and against some influence which holds uric acid in the tissues. Second, with increasing concentration of uric acid in plasma, an increasing proportion entered the tissues. The percentage in tissues increased from about 40 per cent to about 80 per cent, at approximately which proportion an apparent equilibrium was reached. Third, 24 hours after the release of retention, by means of mixed diet, only

2 per cent of the uric acid yet in the body was in plasma. This speaks for a lowered renal threshold.

Although, as we have seen, most of the retained uric acid entered the tissues, yet, because of the great volume of tissue water, the increased concentration of uric acid in tissues was much less than the increased concentration in the plasma. The concentration of uric acid in W. L's plasma increased 9.1 mg. during fasting, whereas concentration in body water outside the plasma increased only 2.76 mg. That is, increased concentration in tissues was 30 per cent of the increase in plasma. This is double the 15 per cent

TABLE VI.

*Proportion of Retained Uric Acid in Plasma at Various Periods in Fasting, with Reference to Uric Acid Level in Plasma.*

Healthy subject, W. L. Data from Tables I and V.

| End of specified days of experiment. | Uric acid in blood plasma. | Retained uric acid found in plasma. | |
|---|---|---|---|
| | | Found. | Corrected.* |
| | *mg. per 100 cc.* | *per cent* | *per cent* |
| 1st day of fast. | 5.48 | 63 | 63 |
| 2nd " " " | 9.30 | 41 | 42 |
| 3rd " " " | 11.27 | 34 | 35 |
| 6th " " " | 14.81 | 30 | 33 |
| 9th " " " | 11.34 | 14 | 18 |
| 12th " " " | 11.2± | 10 | 16 |
| 14th " " " | 12.90 | 11 | 18 |
| 2nd " " food. | 5.00 | 1 | 2 |
| 6th " " " | 2.91 | 0 | 0 |

* Approximate correction for 767 mg. of uric acid in tissues which were not excreted subsequently, and may have been destroyed.

maximum which Folin and his associates obtained in muscles after intravenous injections. In explanation of this difference, it is to be remembered that in fasting, the retention process is slow and accompanied by physiological changes in the tissues, which may make them more permeable to the passage of uric acid. Also an undue proportion of retained uric acid may have been held in kidneys.

*Distribution between Plasma and Cells in the Blood.*

Further information concerning the permeability of tissue cells to uric acid may be gained by a study of the relative concentration

of uric acid in whole blood and plasma. When uric acid is added to blood (Theis and Benedict, 16), or injected (Underhill, 17), most of it remains in the plasma. As viscosity of blood decreases, the relative concentration of uric acid in serum increases (Rouzaud and Thiéry, 18). In toxemia of pregnancy the increase of uric acid in plasma is greater than in the cells (Plass, 19). The rapidity and magnitude of the alterations in concentration of uric acid in blood during fasting experiments gave good opportunity for confirming the observations quoted.

Table VII gives a summary of 84 parallel measurements of uric acid in whole blood and plasma. Contrast would be greater if comparison were made between cells and plasma. In the group with subnormal blood uric acid (less than 3 mg. per 100 cc. of

TABLE VII.

*Distribution of Uric Acid in Whole Blood and Plasma, with Reference to Increasing Concentrations in Whole Blood.*

| Uric acid in whole blood. | No. of specimens. | Average mg. per 100 cc. | | Increase of plasma over whole blood. |
|---|---|---|---|---|
| | | Whole blood. | Plasma. | |
| *mg. per 100 cc.* | | *mg.* | *mg.* | *per cent* |
| Less than 3. | 9 | 2.25 | 2.12 | −5.8 |
| 3–5.5 | 25 | 3.78 | 4.34 | 14.8 |
| 5.6–9.9 | 27 | 7.69 | 9.75 | 26.8 |
| 10 and over. | 18 | 11.45 | 14.48 | 27.0 |
| All cases. | 84 | 6.29 | 7.74 | 23.0 |

whole blood) the concentration in the plasma was 5 per cent less than in whole blood. In the group with normal uric acid content (3 to 5.5 mg. per 100 cc. of whole blood) concentration in plasma was 15 per cent greater than in whole blood. In the two groups with abnormally high blood uric acid this percentage is nearly doubled (27 per cent). We saw from the data presented in Table VI that maintainance of a high level of uric acid in the plasma for a long period did not increase the *proportion* of uric acid in the tissues. So here, the proportion of uric acid in plasma is the same whether uric acid level in the blood is moderately high (5.6 to 9.9 mg.) or very high (more than 10 mg. per 100 cc. of whole blood). It would seem that at the higher levels, there is some degree of equilibrium established between concentration of uric acid in extracellular and intracellular water.

*Alterations in Amounts of Uric Acid in Blood and Tissue.*

In the preceding section we have shown that at high levels of uric acid in the blood there is some evidence of equilibrium between concentration of uric acid in blood and tissues. On the other hand, there are many instances in the accompanying tables, of considerable alteration in the relative amounts of uric acid in blood and tissues. The fasting period which follows (Table VIII and Fig. 7) affords a good example. This patient's urine, because of its small volume, probably could carry no more uric acid. At any rate, uric acid output was fairly constant from day to day. There was no reason to suspect violent changes in production or destruction of uric acid, hence, for each marked fall or rise in blood uric acid, there must have been reciprocal variations in the content of uric acid in the tissues. On the 9th day of fasting concentration of uric acid in the blood fell 8.9 mg. per 100 cc., a 200 per cent change as compared with the normal concentration. This meant a decrease of approximately 506 mg. in the amount of uric acid in the blood, of which 75 mg. went out in the urine and the remaining 431 mg. must have entered the tissues, raising the concentration of uric acid by about 1 mg. per 100 cc. of tissue water, a 50 per cent change as compared with the approximate normal concentration. 4 days later the process was reversed. The blood gained approximately 342 mg., 26 of which came from decreased excretion and 318 from the tissues. In the last 2 days of observation, there was a third variation in relationships, an increased excretion of 914 mg. above the endogenous output, which, with an increase of 93 mg. in the uric acid content of the blood, meant a total of about 1000 mg. of uric acid taken from the tissues, which is probably as much uric acid as the tissues of this individual normally held. As will be seen later, the 24 hour amounts of uric acid excreted under the influence of small amounts of food, may be increased as much as 250 per cent, without change in the level of uric acid in the blood.

Quoting Folin and his coworkers (15) again, ". . . . uric acid is not subject to free distribution between blood and tissues. The uric acid in the muscles, according to our interpretation, is the uric acid actually produced there and bears no relation to the uric acid in the circulating blood. The uric acid produced in

TABLE VIII.

*Forced Concentration of Urinary Uric Acid Throughout Fasting by Means of Limitation of Fluid Intake.*

**Epileptic subject. F. D., male, age 21, weight 64 kilos.**

| Diet. | Day. | Blood. | | Urine. | | | | Concentration of uric acid of urine / blood* |
|---|---|---|---|---|---|---|---|---|
| | | Uric acid per 100 cc. | Plasma bicarbonate. | Volume. | Uric acid. | | | |
| | | | | | Per 100 cc. | 24 hr. output. | Total N. | |
| | | *mg.* | *vol. per cent $CO_2$* | *cc.* | *mg.* | *cc.* | *gm.* | |
| Regular. | Average of 4 days..... | 4.2 | 65.3 | 1400 | 42 | 590* | 10.7 | 10.0 |
| Fast. | 1 | 4.5 | 63.8 | 1350 | 24 | 324 | | 5.3 |
| " | 2 | | 59.0 | 1000 | 39 | 390 | 11.7 | |
| " | 3 | 7.3 | 55.2 | | | | | |
| " | 4 | 6.2 | | 890 | 50 | 445 | 12.9 | 8.0 |
| " | 5 | 7.7 | 55.9 | 610 | 74 | 452 | 11.6 | 9.6 |
| " | 6 | 13.3 | 60.2 | 740 | 47 | 353 | 12.5 | 3.5 |
| " | 7 | 8.4 | 58.5 | 570 | 80 | 455 | 10.8 | 9.5 |
| " | 8 | 16.0<br>16.0 | 60.7 | 640 | 61 | 390 | 12.0 | 3.5 |
| " | 9 | 15.6 | 59.5 | 500 | 93 | 465 | 9.9 | 6.0 |
| " | 10 | 6.7 | | 550 | 74 | 410 | 9.9 | 11.0 |
| " | 11 | 7.8 | 59.8 | 510 | 82 | 418 | 8.9 | 10.5 |
| " | 12 | 6.4 | 57.2 | 470 | 83 | 392 | 9.9 | 6.7 |
| " | 13 | 12.3 | 59.3 | 450 | 76 | 340 | 9.9 | 6.1 |
| Cream. | 1 | 9.4<br>9.2 | 59.0 | 410 | 83 | 342 | 8.6 | 9.0 |
| " | 2 | 8.1<br>9.4 | 63.0<br>57.4 | 400 | 125 | 500 | 9.9 | 14.4 |
| Purine-free. | 1 | 10.2 | 68.3 | 840 | 62 | 525 | 8.2 | 6.0 |
| " | 2 | 5.9 | 69.0 | 640 | 111 | 710 | 10.7 | 19.8 |
| " | 3 | 3.9 | 73.0 | 850 | 57 | 485 | 12.6 | 14.6 |
| " | 4 | 3.6 | 68.5 | 840 | 30 | 259 | 5.9 | 8.2 |
| " | 5 | 3.0 | 68.8 | 1330 | 55 | 740 | 5.1 | 18.4 |
| " | 6 | 4.6 | | 1280† | 83 | 1070 | 3.8 | 18.0 |

* Diet not purine-free. Calculated endogenous output 448 mg.
† Urine for 17 hr. period.

muscles, must, of course, get out, but it gets out not by diffusion but by excretion . . . . ."

Folin is speaking of the comparatively normal physiological condition of the body which attends the intravenous injection of uric acid. It is of interest to know that associated with the metabolic changes in the tissues which attend fasting there may be a considerable movement of uric acid from blood to tissues and *vice versa*. In 24 hours uric acid content of blood, as we have

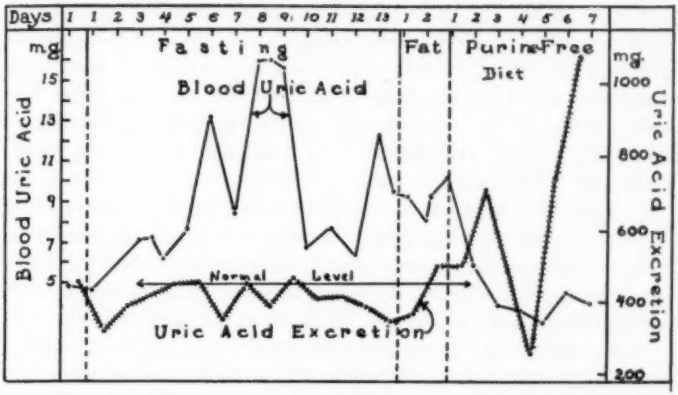


FIG. 7. (Illustrating a part of Table VIII.) 13 day fast of epileptic subject F. D. with limited fluid intake, showing the absence of the usual marked decrease in uric acid excretion. There were unusually violent fluctuations of concentration of uric acid in blood without coincident variations in uric acid output. On the last day of observation there was great increase in uric acid excretion without coincident decrease of uric acid in the circulating blood.

seen, may vary by 200 per cent and of tissues by 50 per cent from the usual normal content. It is true, however, that even these movements, when compared with the ease with which other metabolites move from blood to tissues, are insignificant.

### *Factors Affecting the Retention of Uric Acid.*

In an endeavor to throw light on the cause of the retention, a number of factors were investigated.

*The Ability of the Kidneys to Concentrate Uric Acid.*

Attention was first directed to the kidneys. It was found that decreased excretion of uric acid was attended not by a decreased volume of urine, but by a decrease in the concentration of uric acid in the urine. Under ordinary conditions, the kidney concentrates uric acid ten to thirty times. During fasting this ratio fell to five, three, or even unity. On several occasions (as in Table I, 3rd day) there was actually a lower concentration of uric acid in the 24 hour urine than in the circulating plasma. This remarkable observation immediately raises the question whether the urine, because of its increased acidity, is unable to dissolve uric acid to the usual concentration. To determine this point, uric acid was added to many of these urine samples (Haskins, 20). It was found that they were able to dissolve much more uric acid than they held.

The question next arose: Are these kidneys able to concentrate uric acid to the normal extent? It was evident that if there were such inability, it was not constant. The kidneys which did not concentrate at all at certain times during fasting (Table I) concentrated uric acid as many as 65 times in the postfasting period.

But even during fasting the kidneys could be made to concentrate. Subject F. D. drank only 700 to 1000 cc. of water a day and consequently passed small amounts of urine (Table VIII and Fig. 7). The last column of the table shows that under these conditions the kidneys concentrated uric acid as many as eleven times. Two other subjects drank little water during 1 and 2 day periods, with like results (Tables XVI and XVII).

The disability of the kidneys must lie in lack of ability to absorb uric acid, as Folin and associates suggest for gout, rather than in lack of ability to concentrate it.

Unexpectedly enough, the data show that not only was concentration increased with small volumes of urine, but total output of uric acid was increased also. Thus F. D. passed smaller volumes of urine and maintained a higher uric acid excretion than any other subject. The two other subjects, also, excreted more uric acid on days when fluid intake was limited. This paradoxical relationship does not hold under ordinary conditions of diet, and these three instances are not enough to prove it for fasting.

The findings are hard to explain on the basis of current conceptions of kidney function. They may explain, however, why the decrease in excretion of uric acid during fasting has not attracted more attention. The subject who was carefully studied by Benedict (21) showed little decrease in uric acid excretion after the first few days. His fluid intake and urine volumes were about the same as in this subject F. D.

During fasting there was no decrease in the ability of the kidneys studied to pass phenolphthalein, nitrogen, urea, or a urine of high specific gravity.

*Effect of Uric Acid Injections.*

We wished to determine whether extra uric acid introduced into the body during fasting would be excreted, or would raise the level of uric acid in the blood yet higher. Folin and his associates (15) and Koehler (22) used preparations of uric acid which are much less objectionable than the old solutions. We used the piparazine solution which caused venous thrombosis, hence only six intravenous injections were made. This number was sufficient to demonstrate that intravenous injection of uric acid caused increase in the already high level of uric acid for a few hours only. Table IX shows also, that a smaller proportion of the injected uric acid was excreted during fasting than when the subject was on a purine-free diet. The injections were too few, and the 48 hour observation period too brief to prove whether the uric acid injected during fasting was retained in the tissues or destroyed.

*Effect of Various Food Factors.*

Breaking fast by means of a mixed diet resulted in immediate release of uric acid retention. It seemed important to determine the effect upon the retention of various amounts of pure fat, carbohydrate, and protein.

*Effect of Fat.*

Fat, in the form of 40 per cent cream had no effect upon the retention. Subject J. A. B. (Table X) fasted for 5 days, then took only 40 per cent cream for 4 days (1400 calories 2 days, and 2100 calories 2 days). Blood uric acid remained practically the same

throughout, but fell to normal on resumption of a mixed diet, which furnished fewer calories than the cream. That a high fat, as well as an almost pure fat, diet will maintain retention was shown in the case of L. R. (Table XIX, Fig. 11).

It was found on consulting the literature that a number of workers had encountered a decreased elimination of uric acid with a high fat diet; *viz.*, Horbaczewski and Kanera (23), Herrmann (24), Graham and Poulton (25), who suggested decreased synthesis of purines, Raiziss, Dubin, and Ringer (26) who thought the low excretion represented the true endogenous level, and

TABLE IX.

*Proportion of Injected Uric Acid Recovered from Subjects While Fasting and Not Fasting.*

| Subject | Epileptic, S. R. | | Epileptic, E. B. | | Epileptic, M. C. | |
|---|---|---|---|---|---|---|
| Endogenous excretion, *mg.* | 450* | | 524 | | 510 | |
| Diet | Purine-free. | 8th day fasting. | Purine-free. | 6th day fasting. | Purine-free. | 15th day fasting. |
| | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* |
| Uric acid injected | 800 | 800 | 800 | 800 | 830 | 1000 |
| Uric acid excretion. 1st day | 744 | 691 | 1010 | 492 | 1010 | 492† |
| 2nd day | 578 | 483 | 706 | 653 | 637 | |
| | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| Recovered. 1st day | 37 | 30 | 61 | −4 | 60 | −2 |
| 2nd day | 16 | 4 | 35 | 32 | 15 | |
| Total recovered | 53 | 34 | 96 | 28 | 75 | |

* Assumed as 7 mg. per kilo of body weight.

† Completeness of urine collection questionable.

Umeda (27) who suggested an acidosis which either checked elimination or destroyed uric acid. Lewis and Corley (28) did not find decreased excretion because they collected the urine for only 7 hours after giving the fat. Leopold, Bernhard, and Jacobi (29) found decreased excretion, but no increase of uric acid in the blood drawn on the 2nd day of the diet. Harding, Allin, Eagles, and Van Wyck (30) found increase of uric acid in the blood with a submaintainance, high fat diet, but did not measure uric acid excretion. Their suggested explanation, decrease in volume of blood, will be discussed later.

F. G. Benedict (21) has reviewed the literature in regard to decreased uric acid excretion during fasting. To his list should be added the observations of Zeman and Howe (31) and Leopold *et al.* (29). Benedict concluded that the low and fluctuating level of uric acid excretion during fasting could not be explained on any of the present hypotheses as to its origin. As we have pointed out here and in a previous communication (1) the decreased uric acid output during fasting is due to retention of uric acid within the body. It seems reasonable that the ingestion of fat and the consumption of body fat in fasting should produce similar results. It is believed that the two conditions, in so far as they relate to uric acid, are identical, and that high fat diet, as well as fasting, results in uric acid retention. This relationship will be discussed in more detail later.

TABLE X.

*Effect of Fat Food on Uric Acid Retention during Fasting.*

Epileptic subject. J. A. B., male, age 35, weight 60 kilos.

| Diet. | Day. | Blood. | | | Urine. | | | |
|---|---|---|---|---|---|---|---|---|
| | | Per 100 cc. | | Plasma bicarbonate. | Volume. | Uric acid. | | Total N. |
| | | Uric acid. | Non-protein N. | | | Per 100 cc. | 24 hr. output. | |
| | | *mg.* | *mg.* | *vol. per cent $CO_2$* | *cc.* | *mg.* | *mg.* | *gm.* |
| Fast. | 1 | 3.26 | 35.3 | | 670 | 53.5 | 359 | 6.09 |
| " | 2 | 4.22 | 38.7 | 65.1 | 510 | 56.2 | 287 | 9.02 |
| " | 3 | 6.05 | 42.8 | | 1075 | 24.7 | 268 | 15.30 |
| " | 4 | 7.14 | 61.0 | | 1460 | 45.4 | 663 | 13.90 |
| " | 5 | | | | 840 | 35.3 | 297 | 15.90 |
| 40 per cent cream (1400 calories.) | 1 | 7.62 | 40.0 | 58.0 | 700 | 38.4 | 269 | 10.60 |
| " " | 2 | 7.26 | 44.1 | 52.8 | 600 | 68.6 | 412 | 8.16 |
| (2100 calories.) | 3 | 6.72 | 41.4 | 59.0 | 530 | 39.6 | 209 | 4.45 |
| " " | 4 | 6.72 | 35.3 | 57.8 | 790 | 37.3 | 295 | 7.60 |
| Mixed. | 1 | 7.55 | 34.9 | 62.7 | 1384 | 61.2 | 847 | 5.48 |
| | | 6.45 | 31.6 | 67.5 | | | | |
| " | 2 | 4.45 | 37.1 | 68.2 | 550 | 73.5 | 404 | 5.50 |
| " | 3 | | | | 1350 | 46.5 | 630 | 9.00 |
| " | 4 | 2.54 | 35.9 | 70.8 | 1660 | 44.5 | 738 | 6.50 |
| " | 5 | 3.27 | 38.7 | 63.5 | 1100 | 51.0 | 563 | 9.37 |

*Effect of Carbohydrates.*

Ingestion of carbohydrates in the form of glucose and orange juice, and in amounts as small as 100 to 125 gm. promptly released uric acid retention (Table XI; see also Table XV and Fig. 8).

TABLE XI.

*Effect of Carbohydrate Food on Uric Acid Retention during Fasting.*

| Epileptic subjects. | Diet. | Day. | Uric acid. | |
|---|---|---|---|---|
| | | | Blood per 100 cc. | Urine 24 hr. excretion. |
| | | | *mg.* | *mg.* |
| S. H., male, age 30, weight 50 kilos. | Fast. | 11th | 7.3 | 352 |
| | " | 12th | 6.5 | 328 |
| | " | 13th | 8.0 | 500 |
| | Orange juice (carbohydrate 95 gm., 420 calories). | 1st | 9.4 | 692 |
| | Orange juice (carbohydrate 95 gm., 420 calories). | 2nd | 5.4 | 717 |
| | Purine-free. | 1st | 5.0 | 558 |
| | " | 2nd | 3.9 | 458 |
| | " | 3rd | 3.6 | 295 |
| I. S., female, age 22, weight 39 kilos. | Fast. | 5th | 6.1 | 114 |
| | " | 6th | 5.3 | 137 |
| | Glucose and oranges: (carbohydrate 135 gm., 570 calories). | 1st | 5.9 | 755 |
| | (carbohydrate 266 gm., 1080 calories). | 2nd | 3.7 | 365 |
| | (carbohydrate 135 gm., 548 calories). | 3rd | 4.1 | 171 |

Lewis and Corley (28) attributed increased excretion of uric acid following ingestion of honey to stimulation of cellular metabolism. Leopold *et al.* (29) failed to find increase. Rémond and Rouzaud (32) found a decrease of uric acid in blood as well as increased excretion following intravenous injection of glucose in patients with gout. It seems evident that the extra uric acid

eliminated in the experiments of Lewis and Corley, was simply uric acid already present in blood and tissues.

*Effect of Protein and Amino Acids.*

Much has been written about the effect of protein feeding on uric acid elimination. Folin *et al.* (15) have reviewed the literature. Besides the authors mentioned by them, it should be noted that Raiziss *et al.* (26), Rose (33), and Leopold *et al.* (29) have reported increased elimination of uric acid with high protein feeding.

In addition to this evidence, Lewis, Dunn, and Doisy (34) found that the ingestion of various amino acids caused prompt increase in uric acid output. They think this increase is "the result of a stimulation of uric acid production rather than of a more rapid excretion of the uric acid already present in the system, since successive doses of glycocoll on the same experimental day resulted in an increased elimination of uric acid in each case." This reasoning is not conclusive. Increased elimination of many times the 10 to 20 mg. which they found would not deplete the store of uric acid already in the system. On this reasoning, one must conclude that the use of atophan causes increased production of uric acid, because successive doses result in an increased elimination of uric acid in each case.

By feeding large amounts of the amino acids during fasting, if Lewis' theory was correct, we hoped to cause excretion of a larger amount of uric acid than could be accounted for by the amount present in the body. Two experiments (Table XII) were made with glycine (glycocoll). Ingestion of 40 and 20 gm. by two subjects increased the 24 hour uric acid elimination by 250 and 98 per cent respectively, with practically no change in the uric acid content of the blood.

Three experiments were made with asparagine (Table XIII and Table XV, Fig. 8). Feeding of 33, 30, and 25 gm. of asparagine resulted in increased elimination of 117, 80, and 117 per cent of uric acid without any decrease in the level of circulating uric acid.

The result with E. B., Table XIII, was the most marked of any of the five experiments. Following the ingestion of 33 gm. of asparagine, the 24 hour output of uric acid was increased by 385 mg. During the same period the blood gained 3.5 mg. per

100 cc., or 164 mg. for the whole volume of blood. This meant a total of 549 more mg. of uric acid in blood and urine. Therefore, during this 24 hour period, 33 gm. of asparagine either more than doubled the usual endogenous production of uric acid or took 549 mg. from the tissues, reducing the concentration of uric acid in tissue water by approximately 1.6 mg. per 100 cc. If the subject were on normal diet, the withdrawal of this amount

TABLE XII.

*Effect of Glycine on Uric Acid Retention during Fasting.*

| Epileptic subjects. | Day. | Remarks. | Blood. | | | | Urine. | |
|---|---|---|---|---|---|---|---|---|
| | | | Hr. taken. | Per 100 cc. | | | | |
| | | | | Uric acid. | Amino acid N. | Non-protein N. | Uric acid 24 hr. output. | Total N. |
| | | | | *mg.* | *mg.* | *mg.* | *mg.* | *gm.* |
| M. M., male, age 21, weight 55 kilos. | 7th | | 10.00 a.m. | 8.82 | | 41.3 | 188 | 8.60 |
| | 8th | Glycine 20 gm. 10.30 a.m.–12 p.m. | 10.30 " | 9.61 | | 40.8 | | |
| | | Glycine 10 gm. 2–4 p.m. | 2.00 p.m. | 9.67 | 5.13 | | | |
| | | Glycine 10 gm. 6–8 p.m. | 6.00 " | 9.08 | 4.66 | | 663 | 11.21 |
| | 9th | | 10.00 a.m. | 8.43 | 4.37 | | 240 | 7.68 |
| F. R., male, age 20, weight 55 kilos. | 5th | | 10.30 a.m. | 7.46 | | 34.7 | 175 | 7.13 |
| | 6th | | 6.00 p.m. | 9.67 | | 40.5 | 175 | 6.25 |
| | 7th | Glycine 20 gm. 10.05 a.m.– 7.00 p.m. | 10.00 a.m. | 9.55 | 5.28 | 37.8 | | |
| | | | 2.00 p.m. | 9.13 | 5.60 | | | |
| | | | 6.00 " | 9.52 | 6.02 | | 346 | 7.35 |
| | 8th | | 10.00 a.m. | 9.22 | 5.87 | | 223 | 7.00 |

of uric acid from his tissues would nearly drain them, but because he had been fasting for 11 days, this amount probably represents only a part of the uric acid retained during his fast. In order to demonstrate increased production, one would need to use much larger quantities of the amino acids than was deemed feasible. The use of protein, however, should serve the same purpose. There is no reason to believe that the action of protein with re-

spect to possible stimulation of metabolism is different from its constituent amino acids.

Three of the subjects broke fast by eating white of egg (Table XIV; see also Table XV). Small amounts of protein, up to 96 gm. a day (like the amino acids) caused increased output of uric acid without affecting the level in the blood. Subject E. B. in 2 days was able to consume the whites of 75 eggs, equivalent to 240 gm.

TABLE XIII.

*Effect of Asparagine on Uric Acid Retention during Fasting.*

| Epileptic subjects. | Day. | Remarks. | Blood. | | | Urine. | |
|---|---|---|---|---|---|---|---|
| | | | Hr. taken. | Uric acid per 100 cc. | Amino acid N. | Volume. | Uric acid 24 hr. output. |
| | | | | *mg.* | *mg.* | *cc.* | *mg.* |
| E. B., male, age 28, weight 51 kilos. | 11th | | 10.00 a.m. | 8.33 | | 2200 | 329 |
| | 12th | Asparagine 33 gm., in 1100 cc. water from 12.05–6 p.m. | 12.00 p.m. | 8.78 | 4.67 | | |
| | | | 6.00 " | 11.40 | 5.27 | 1820 | 714 |
| | 13th | | 10.00 a.m. | 12.30 | 4.92 | 1400 | 539 |
| I. S., female, age 22, weight 39 kilos. | 3rd | | 10.00 a.m. | 5.92 | | 525 | 125 |
| | 4th | Asparagine 10 gm., 10–12 a.m. | 1.30 p.m. | 7.33 | | | |
| | | Asparagine 10 gm., 2–5 p.m. | 7.00 " | 6.35 | 5.00 | | |
| | | Asparagine 10 gm., 7–9 p.m. | | | | 1810 | 229 |
| | 5th | | 2.00 p.m. | 6.06 | | 570 | 114 |

of protein, about a third of his caloric requirement. During the 2nd day, the egg albumen taken contained 144 gm. of protein. On this day the urine contained 966 mg. of uric acid, and uric acid in the blood fell from 10 mg. to 5.1 mg. per 100 cc. This experiment proved that pure protein, as well as pure carbohydrate, released uric acid retention.

In other experiments even with large amounts of protein we never got a larger uric acid output than could be accounted for by elimination of uric acid already in the tissues. In one experi-

TABLE XIV.

*Effect of Protein Food on Uric Acid Retention during Fasting.*

| Epileptic subjects. | Diet. | Day. | Blood. Per 100 cc. Non-protein N. | Blood. Per 100 cc. Uric acid. | Urine. 24 hr. excretion. Uric acid. | Urine. 24 hr. excretion. Total N. |
|---|---|---|---|---|---|---|
| | | | *mg.* | *mg.* | *mg.* | *gm.* |
| E. B., male, age 28, weight 51 kilos. | Fast. | 13 | | 12.30 | 539 | |
| | Egg albumen (96 gm. protein = 390 calories). | 1 | 28.6 | 10.00 | 707 | 10.5 |
| | Egg albumen (144 gm. protein = 590 calories). | 2 | 38.8 | 10.00 | 966 | 17.2 |
| | Glucose (81 gm. carbohydrate = 332 calories). | 1 | | 5.16 | 432 | 11.5 |
| | Purine-free. | 1 | 41.4 | 4.32 | 550 | 7.5 |
| | " | 2 | | 3.34 | 738 | |
| Bel., female, age 21, weight 49 kilos. | Purine-free. Average 3 days. | | | 3.9 | 314 | |
| | Fast. | 1 | | 3.2 | 149 | |
| | " | 2 | | | 111 | |
| | " | 3 | | 4.3 | 130 | |
| | " | 4 | | | 150 | |
| | " | 5 | | 6.4 | 152 | |
| | " | 6 | | 8.9 | 244 | |
| | Egg albumen (38 gm. protein = 155 calories). | 1 | | 8.3 | 276 | |
| | Egg albumen (28 gm. protein = 115 calories). | 2 | | 8.3 | 313 | |
| | Egg albumen (28 gm. protein = 115 calories). | 3 | | 8.3 | 276 | |
| | Fast. | 1 | | | 239 | |
| | Purine-free. | 1 | | 7.3 | 545 | |
| S. R., male, age 32, weight 64.3 kilos. | Fast. | 14 | | 8.3 | 270 | |
| | Egg albumen (57 gm. protein = 228 calories). | 1 | | 9.4 | 347 | |
| | Egg albumen (96 gm. protein = 390 calories). | 2 | | 11.4 | 608 | |
| | Purine-free. | 1 | | 11.8 | 807 | |
| | " | 2 | | 5.2 | 665 | |

ment uric acid in blood and urine were measured for a period of 35 days after fasting. During 15 of these days the subject ate a constant diet of lean meat and white of eggs only. During the first 5, when no salt was taken, the daily uric acid output averaged only 33 mg. above the endogenous level. With the addition of 10 gm. of salt to this protein diet the output for 10 days averaged 114 mg. above the endogenous level. Making allowance for the fact that the meat diet, which contained liver, was not purine-free, the increased uric acid excretion with 5 days of protein, and 10 days of protein and salt feeding, was no greater than the amount retained during 6 days of fasting.

If protein feeding stimulates uric acid production and in that way causes increased uric acid output, the increased elimination should continue as long as protein feeding continues. Rose (33) and Leopold (35) have found that on continued feeding of high protein diet, the uric acid output, which was high at first, later declined to the endogenous level. Leopold attributes this decline to kidney "fatigue." Rather it would seem that the limit had been reached of uric acid in blood and tissues available for excretion.

All our evidence, then, is against the views expressed by Lewis and his associates, and in favor of a slow storage of uric acid under the influence of incomplete fat metabolism, and a comparatively quick elimination of uric acid contained in tissue and blood, under the influence of carbohydrate and protein metabolism.

*Comparative Effect of Various Foods.*

We attempted to compare the effect of these foods by feeding amounts of fat, protein, and carbohydrate sufficient to supply caloric requirements for about 4 hours to a healthy fasting subject. Urine was collected and 200 cc. of water were drunk every 4 hours. Blood was drawn thirty-three times during the 9 days of the experiment (Table XV, Fig. 8).

It is noticeable, in the first place, that the extra elimination of uric acid which followed the ingestion of asparagine, glucose, and albumen (except 94 of the 274 mg. which followed ingestion of albumen) came from tissues and not from blood. The fact that there was more than 100 per cent increase in uric acid elimination without change in the level of uric acid in the blood disproves the

assertion of Höst (36) that if blood uric acid remains the same, variation in output must mean variation in production.

TABLE XV.

*Comparative Effect of Various Types of Food on Uric Acid Retention during Fasting.*

Healthy subject. W. L., male, age 38, weight 64 kilos.
Water intake, 200 cc. every 4 hrs.

| Blood. | | Urine. | | | | | | Remarks. |
|---|---|---|---|---|---|---|---|---|
| | | | | Uric acid. | | | | |
| Hr. taken. | Uric acid per 100 cc. | Period. | Volume. | Per 100 cc. | Total output. | Total N. | pH | |
| | mg. | | cc. | mg. | mg. | gm. | | |
| Apr. 6 | | | | | | | | |
| 8.00 p.m. | 3.2 | 8–12 p.m. | 170 | 49 | 84 | 1.60 | | |
| Apr. 7 | | 12– 4 a.m. | 180 | 32 | 58 | 1.51 | | |
| | | 4– 8 " | 130 | 50 | 66 | 1.21 | | |
| 10.30 a.m. | 3.2 | 8–12 " | 440 | 26 | 113 | 1.81 | | |
| 1.10 p.m. | 3.2 | 12– 4 p.m. | 280 | 25 | 71 | 1.40 | | |
| 4.40 " | 3.6 | 4– 8 " | 105 | 36 | 38 | 0.89 | | |
| 11.00 " | 4.1 | 8–12 " | 120 | 27 | 32 | 1.12 | | |
| Apr. 8 | | 12– 4 a.m. | 280 | 8 | 25 | 1.46 | | |
| | | 4– 8 " | 220 | 10 | 21 | 1.72 | | |
| 10.20 a.m. | 4.65 | 8–12 " | 180 | 14 | 26 | 1.91 | | |
| 12.00 n. | 4.7 | 12– 4 p.m. | 240 | 8 | 20 | 2.41 | | At 12.25 p.m. cream.* |
| 4.15 p.m. | 4.9 | 4– 8 " | 130 | 18 | 24 | 1.83 | | |
| 8.00 " | 8.3 | 8–12 " | 144 | 13 | 18 | 2.03 | | |
| Apr. 9 | | 12– 4 a.m. | 172 | 11 | 20 | 1.96 | | |
| | | 4– 8 " | 270 | 10 | 27 | 2.51 | | |
| 10.30 a.m. | 5.2 | 8–12 " | 190 | 14 | 28 | 1.90 | | |
| | | 12– 4 p.m. | 180 | 16 | 29 | 2.02 | 5.6 | |
| 4.00 p.m. | 5.8 | 4– 8 " | 192 | 33 | 63 | 2.76 | 6.1 | At 4.05 p.m. asparagine.† |
| 8.00 p.m. | 5.7 | 8–12 " | 184 | 13 | 24 | 2.42 | 6.0 | |
| Apr. 10 | | 12– 4 a.m. | 275 | 10 | 27 | 2.64 | 6.1 | |
| | | 4– 8 " | 260 | 10 | 26 | 1.58 | 6.1 | |
| 11.45 a.m. | 7.8 | 8–12 " | 125 | 26 | 32 | 1.28 | 5.9 | |
| 3.00 p.m. | 6.7 | 12– 4 p.m. | 150 | 37 | 56 | 1.97 | 5.6 | At 12.00–1.45 p.m. white of egg.‡ |
| 4.30 " | 6.2 | 4– 8 " | 140 | 44 | 62 | 1.90 | 5.9 | |
| 9.00 " | 6.4 | 8–12 " | 122 | 60 | 74 | 1.65 | 6.1 | |

TABLE XV—*Concluded.*

| Blood. | | Urine. | | | | | | Remarks. |
|---|---|---|---|---|---|---|---|---|
| Hr. taken. | Uric acid per 100 cc. | Period. | Volume. | Uric acid. Per 100 cc. | Uric acid. Total output. | Total N. | pH | |
| | *mg.* | | *cc.* | *mg.* | *mg.* | *gm.* | | |
| Apr. 11 | | 12– 4 a.m. | 124 | 53 | 66 | 1.57 | 6.1 | |
| | | 4– 8 " | 110 | 76 | 83 | 1.57 | 6.2 | |
| 11.45 a.m. | 6.2 | 8–12 " | 85 | 110 | 94 | 1.30 | 6.2 | |
| | | 12– 4 p.m. | 125 | 43 | 54 | 2.37 | 5.9 | |
| 4.00 p.m. | 6.9 | 4– 8 " | 160 | 22 | 35 | 2.67 | 5.6 | |
| 9.00 " | 6.7 | 8–12 " | 295 | 8 | 24 | 2.31 | 5.8 | |
| Apr. 12 | | 12– 4 a.m. | 250 | 11 | 30 | 1.95 | 5.6 | |
| 7.45 a.m. | 6.6 | 4– 8 " | 135 | 21 | 32 | 1.75 | 5.6 | |
| 10.00 " | 6.7 | 8–12 " | 160 | 65 | 105 | 2.13 | 5.6 | At 8.00 a.m. glucose.§ |
| 12.00 n. | 6.6 | 12– 4 p.m. | 100 | 58 | 58 | 1.63 | 5.9 | |
| 4.30 p.m. | 6.6 | 4– 8 " | 108 | 51 | 55 | 1.83 | 5.8 | |
| 9.30 " | 6.9 | 8–12 " | 205 | 22 | 46 | 1.64 | 6.0 | |
| Apr. 13 | | 12– 4 a.m. | 288 | 12 | 36 | 1.60 | 6.3 | |
| 8.00 a.m. | 6.7 | 4– 8 " | 175 | 28 | 50 | 1.70 | 6.2 | At 8.15 a.m. mixed purine-free food. |
| 12.15 p.m. | 6.1 | 8–12 " | 120 | 103 | 123 | 1.83 | 6.1 | |
| Apr. 13–14‖ | 4.3 | 12–12 n. | 489 | 145 | 711 | 7.87 | 6.0 | Purine-free food. |
| " 14–15 | 3.7 | 12–12 " | 624 | 88 | 548 | 6.99 | 6.0 | " " |
| " 15–16 | 3.7 | 12–12 " | 747 | 66 | 498 | 8.54 | 6.1 | " " |

* Cream taken 160 cc., containing 54 gm. of fat and 500 calories.

† Asparagine taken, 25 gm., 100 calories.

‡ White of egg taken, containing 85 gm. of protein and 340 calories. 1 to 2 gm. NaCl used on egg.

§ Glucose taken, 85 gm., 340 calories.

‖ To save space, 4 hourly observations for the last 3 days are consolidated into 24 hour periods. 4 hourly data concerning uric acid are shown in Fig. 8.

Consequent on ingestion of the three principal foodstuffs, there was the following increase of uric acid excretion above the fasting level, *viz.* fat 0, glucose 136, albumen 274, or a ratio of 0, 1, and 2, respectively. The fact that albumen had a greater effect than an

equal amount of glucose, is against the possibility that the effect of protein was due to the fraction of glucose which it yielded. Since this is only one observation, it should not be given too much weight.

There was marked contrast, not only in the amount of uric acid eliminated, but also in the length of time during which excretion remained high. Following ingestion of the foods, maxi-

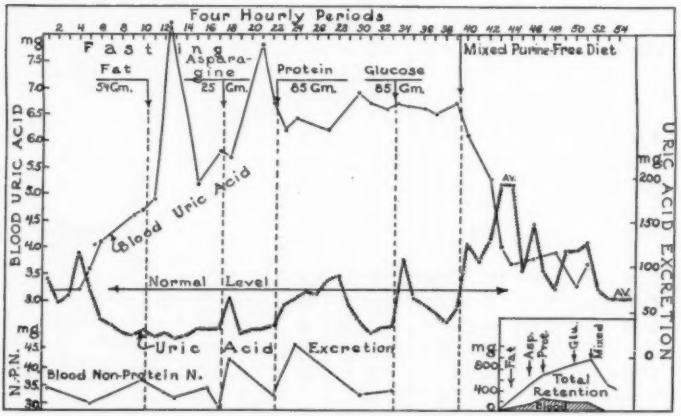


FIG. 8. (Illustrating part of Table XV.) 6 day fast of healthy subject W. L. with observations every 4 hours on blood uric acid and uric acid excretion. The chart shows the comparative effect on uric acid retention of fat (in the form of cream), of asparagine, of protein (in the form of white of egg), of glucose, and of mixed, purine-free diet. The small chart in the lower right corner indicates (as in Fig. 6) the total uric acid retention for the period and the amount of retained uric acid contained in the blood. 63 per cent of the uric acid retained during fasting was excreted in the post-fasting period.

mum excretion occurred within 4 hours in the case of asparagine and glucose, and not till 20 hours in the case of albumen. This lengthened period for protein suggests the longer time required for its digestion and absorption as compared with the quick absorption of amino acids and glucose. These time relations suggest the period over which the specific dynamic action of these foodstuffs (as measured by oxygen comsumption) is manifested. Lewis and Corley (28) state: "The influence of uric acid excretion

runs roughly parallel to the phenomena of specific dynamic action." That these two phenomena are not parallel is shown by the fact that specific dynamic action of fats is as great as that of carbohydrates, yet the former causes decrease, and the latter increase in uric acid excretion. Again, aspartic and glutamic acids, like other amino acids, cause increased elimination of uric acid (Lewis, *et al.*, 34), whereas they exhibit little specific dynamic action (Lusk, 37).

In relation to gout, these observations on the effect of foods suggest that overeating of fat, rather than of protein, is of etiological importance. In relation to treatment, they suggest the use of a low fat diet; *i.e.*, one consisting mainly of carbohydrate and protein.

### *Effect of Atophan.*

Atophan by mouth was given in three of the fasting periods. 3.5 gm. given to subject E. T. (Table XVI, Fig. 9) produced a prompt elimination of the uric acid retained in the blood. During the first 24 hours, the blood lost 375 mg. and the urine gained 301 mg. For the following 2 days, with the aid of 1.5 gm. more atophan, the uric acid in the urine measured 1085 mg. more than the estimated endogenous excretion for that period plus the additional 50 mg. lost from the blood. This gm. or more of uric acid, which must have come from the tissues, was probably a larger amount than the tissues of this subject normally contained. This, then, is additional evidence that uric acid is stored in the tissues during fasting. The increased elimination of uric acid which followed the taking of atophan was not accompanied by diuresis, increased excretion of nitrogen, or any significant changes in plasma bicarbonate of blood or pH of urine. We need to know more about the physiological changes produced in kidneys by this remarkable drug.

An interesting comparison of the effect of glycine and atophan is presented in Table XVII and Fig. 10. (Details of the administration of glycine are contained in Table XII). The 40 gm. of glycine and the 4 gm. of atophan produced an increase in uric acid excretion of 475 mg. and 506 mg. respectively. In the case of atophan there was a coincident fall of blood uric acid to normal, accounting for more than half of the increased excretion.

In the case of glycine, there was practically no loss from the blood, therefore all the uric acid must have come from the tissues.

In this and other experiments in which 4 to 5 gm. of atophan were used, the renal threshold for uric acid was lowered with con-

TABLE XVI.

*Effect of 5 Gm. of Atophan on Uric Acid Retention.*

Epileptic subject. E. T., age 14, weight 58.1 kilos.

| Diet. | Day. | Blood. | | | Urine. | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Uric acid. | Non-protein N. | Plasma bicarbonate. | Volume. | Uric acid. | | Total N. | pH |
| | | | | | | Per 100 cc. | Total output. | | |
| | | *mg.* | *mg.* | *vol. per cent $CO_2$* | *cc.* | *mg.* | *mg.* | *gm.* | |
| Fast.* | 1 | 4.22 | 32.1 | 63.9 | 2400 | 13 | 320 | 7.36 | |
| " | 2 | 4.45 | 34.3 | 57.3 | 1990 | 10 | 203 | 7.10 | |
| " | 3 | 6.30 | 32.5 | 58.7 | 1990 | 10 | 198 | 8.81 | 5.6 |
| " | 4 | 6.16 | 34.0 | 54.9 | 1075 | 21 | 250 | 7.62 | 7.1 |
| " | 5 | 7.17 | 37.0 | 54.7 | 590 | 63 | 375 | 9.29 | 5.6 |
| " | 6 | 9.02 | 36.2 | 64.7 | 1155 | 34 | 392 | 8.26 | 5.7 |
| " † | 7 | 9.85 | 35.7 | 62.8 | 984 | 70 | 693 | 7.00 | 5.9 |
| | | 7.17 | 32.4 | | | | | | |
| " ‡ | 8 | 4.35 | 31.9. | 61.5 | 850 | 72 | 709 | 6.30 | 5.6 |
| " | 9 | 4.00 | 31.6 | 55.9 | 1840 | 66 | 1210 | 7.37 | 5.8 |
| " | 10 | 3.48 | 37.5 | 66.2 | 1660 | 17 | 291 | 7.72 | 5.7 |
| Whites of 12 eggs. | | 3.48 | 32.5 | 68.0 | 1550 | 28 | 440 | 7.95 | 7.3 |
| Purine-free. | 1 | 4.20 | 33.4 | 71.4 | 1370 | 40 | 554 | 8.45 | 5.9 |
| " | 2 | 4.15 | 33.7 | 65.3 | 1850 | 22 | 402 | 7.65 | 6.0 |
| " | 3 | 3.48 | 33.9 | 69.3 | 2530 | 18 | 468 | 8.07 | 7.0 |
| " | 4 | 3.20 | 35.3 | 70.6 | 2325 | 12 | 277 | 5.81 | 6.7 |
| " | 5 | 2.00 | 25.2 | | | | | | |

* The morning of this day ate a dish of rice. Had fasted for 2 days previously.

† Blood taken at 9.30 a.m. Atophan given as follows: 1.5 gm. at 10.00 a.m., 1 gm. at 1.00 p.m. and 4.00 p.m. Second blood taken at 6.00 p.m.

‡ Atophan 1.5 gm. at 9.00 a.m.

sequent drainage of surplus uric acid from the blood, and, after that, drainage of retained uric acid from the tissues. This evidence is against the opinion of Griesbach (38) that atophan increases mobilization of uric acid from the tissues. (It is possible that smaller, single doses of atophan act differently. See Table XIX.)

On the other hand, ingestion of amounts of food ten to twenty times larger than the doses of atophan, first caused a loss of uric acid from tissues. With repeated ingestion of food there followed a lowering of the renal threshold and drainage of uric acid from both blood and tissues. Although this was the usual sequence, variations occurred. For example, during the first 2 days of food of subject S. S. (Table II, Fig. 2) approximately 285 mg. of uric acid disappeared from the blood, with no coincident increase in

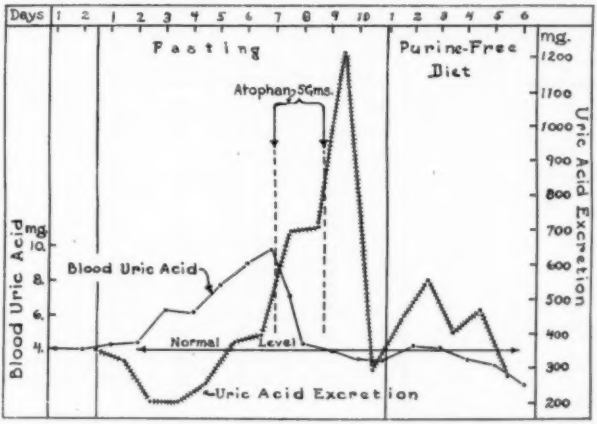


FIG. 9. (Illustrating part of Table XVI.) Fast of epileptic subject E. T. showing that 5 gm. of atophan reduced blood uric acid to normal, by means of increased elimination of uric acid in the urine. The subsequent 24 hour output of 850 mg. above the endogenous level must have come from the tissues.

uric acid excretion. Apparently the uric acid went into the tissues instead of into the urine, because 2 days later there was a greatly increased output of uric acid in the urine.

### *Effect of Thyroid Feeding.*

During fasting there is usually a marked decrease in the basal metabolic rate, the cause of which is not clear. We wished to determine the effect of artificially raising the rate. For this purpose, 54 gr. of thyroid extract were given to the subject F. R. from the 8th to

the 12th day, inclusive, of fasting (Table XVIII). This increased his basal metabolic rate 21 per cent. From the end of the 9th to

TABLE XVII.

*Comparative Effect of Glycine and Atophan on Uric Acid Retention.*

Epileptic subject. M. M., age 21, weight 55 kilos.

| Diet. | Day. | Blood. | | Urine. | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Uric acid per 100 cc. | Plasma bicarbonate. | Volume. | Uric acid. | | Total N. | pH |
| | | | | | Per 100 cc. | 24 hr. output. | | |
| | | *mg.* | *vol. per cent $CO_2$* | *cc.* | *mg.* | *mg.* | *gm.* | |
| Purine-free. | 1 | 3.48 | 66.3 | 1340 | 40 | 535 | 5.06 | 5.8 |
| " | 2 | 3.80 | | 1260 | 39 | 485 | 11.53 | 5.3 |
| " | 3 | 3.08 | | 1370 | 35 | 495 | 10.70 | 5.6 |
| Fast. | 1 | 3.31 | 64.2 | 1100 | 24 | 268 | 8.53 | 5.4 |
| " | 2 | 2.99 | 57.4 | 1500 | 19 | 278 | 10.9 | 5.4 |
| " | 3 | 5.92 | 50.1 | 1340 | 17 | 225 | 10.3 | 5.6 |
| " | 4 | 5.84 | | 1070 | 14 | 151 | 8.04 | 5.3 |
| " | 5 | 8.33 | 55.7 | 410 | 84 | 344 | 7.96 | 5.4 |
| " | 6 | 10.70 | 55.3 | 520 | 48 | 238 | 7.55 | 5.4 |
| " | 7 | 8.82 | 53.4 | 1750 | 11 | 188 | 8.60 | 5.3 |
| " | 8* | 9.61 | 55.6 | 1850 | 35 | 663 | 11.21 | 5.8 |
| " | 9 | 8.43 | 55.1 | 1320 | 18 | 240 | 7.68 | 6.0 |
| " | 10 | 9.52<br>10.00 | 59.2 | 1540 | 29 | 453 | 8.25 | 5.3 |
| " | 11 | 10.2 | 56.5 | 950 | 36 | 349 | 8.26 | 5.5 |
| " | 12† | 10.3 | 63.2 | 1710 | 50 | 855 | 8.22 | 6.0 |
| " | 13 | 4.57 | 55.5 | 1100 | 13 | 145 | 9.57 | 6.0 |
| " | 14 | 5.37 | | 1720 | 6 | 104 | 7.65 | 5.5 |
| Egg albumen. | 1 | 5.85 | 52.5 | 2100 | 11 | 234 | 9.42 | 5.3 |
| " " | 2 | 6.78 | 70.6 | 1840 | 23 | 411 | 9.68 | 5.6 |
| Purine-free. | 1 | 5.76 | 68.3 | 1340 | 51 | 697 | 9.05 | 6.9 |
| " | 2 | 5.33 | 66.5 | 1040 | 50 | 520 | 9.20 | 5.8 |
| " | 3 | 4.00 | 68.5 | 1070 | 46 | 487 | 5.00 | 5.8 |
| " | 4 | 3.85 | 65.7 | 2160 | 18 | 392 | 4.52 | 5.6 |
| " | 5 | 2.96 | 66.5 | 1520 | 56 | 854 | 7.60 | 5.8 |

* 40 gm. glycine. For details see Table X.

† 4 gm. atophan given after taking of blood.

the end of the 14th day of fasting, uric acid in the blood decreased from 10.7 mg. to 6.2 mg. per 100 cc. and uric acid excretion coinci-

dently increased to well above the endogenous level. During the 3 days preceding and the first 5 days of the fast detailed in Table XV, the healthy subject W. L. took 79 gr. of thyroid, thereby increasing his basal metabolic rate about 35 per cent. The concentration of uric acid in the blood did not increase as rapidly in this fast as it had in previous fasts undergone by the same subject.

The evidence from only two experiments cannot be taken as conclusive. Granted that thyroid feeding did cause partial release of the uric acid retention, it is possible that in the artificially stimulated consumption of body material, carbohydrate may have

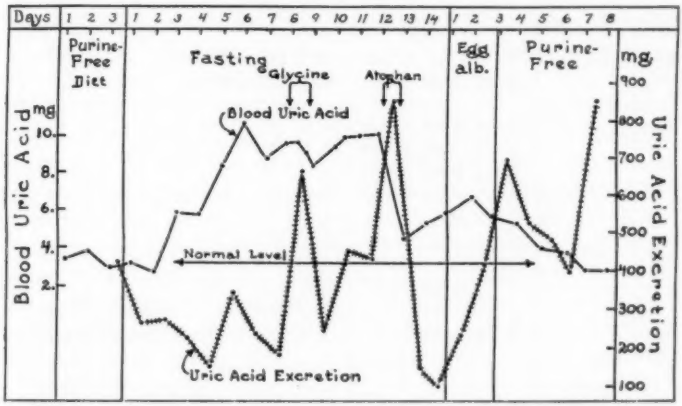


FIG. 10. (Illustrating part of Table XVII.) Fast of epileptic subject M. M. showing that 40 gm. of glycine caused increased uric acid output without lowering level of uric acid in blood in contrast with 4 gm. of atophan which caused about the same increase in uric acid excretion with coincident fall to normal in the amount of uric acid in the blood.

been burned in larger proportion than during simple fasting. Supporting this supposition in the case of F. R., was the disappearance of acidosis, as evidenced by increase of plasma bicarbonate to normal, coincident with decrease of blood uric acid. The lack of increased nitrogen in the urine is against any marked increase in metabolism of protein. Increasing the basal metabolic rate by means of exercise, contrary to seeming results with thyroid, had no tendency to relieve uric acid retention. The relationship of uric acid metabolism to basal metabolic rate deserves further study.

TABLE XVIII.

*Effect of the Administration of Thyroid Extract on Uric Acid Retention.*

Epileptic subject. F. R., age 17, weight 55 kilos.

| Diet. | Day. | Blood. | | Urine. | | | | | Remarks. |
|---|---|---|---|---|---|---|---|---|---|
| | | Uric acid per 100 cc. | Plasma bicarbonate. | Volume. | Uric acid. Per 100 cc. | Uric acid. 24 hr. output. | Total N. | pH | |
| | | *mg.* | *vol. per cent $CO_2$* | *cc.* | *mg.* | *mg.* | *gm.* | | |
| Purine-free. | 1 | 3.81 | 61.2 | 940 | 42 | 498 | 8.17 | 6.0 | |
| " | 2 | 5.33 | 58.2 | 800 | 54 | 433 | 6.50 | 6.1 | |
| Fast. | 1 | 4.85 | 60.2 | 310* | 100.0 | 310 | 4.66 | 6.0 | |
| " | 2 | 5.33 | 62.3 | 2290 | 8.5 | 196 | 9.16 | 5.9 | |
| " | 3 | 5.33 | 53.7 | 2200 | 10.4 | 228 | 8.20 | 5.3 | |
| " | 4 | 6.40 | 56.4 | 1430 | 10.4 | 148 | 7.95 | 5.3 | |
| " | 5 | 7.46<br>9.75<br>9.67 | 47.0 | 1420 | 12.4 | 175 | 7.13 | 5.4 | |
| " | 6 | 9.13 | 50.0 | 1050 | 16.6 | 175 | 6.25 | 5.3 | |
| " | 7 | | 51.2 | 970 | 35.6 | 346 | 7.35 | 5.9 | Glycine 20 gm. |
| " | 8 | 9.22 | 55.5 | 1400 | 20.9 | 223 | 7.00 | 5.0 | Basal metabolism 0 per cent. Thyroid, gr. 12. |
| " | 9 | 10.40 | 53.0 | 720 | 47.7 | 343 | 5.14 | 5.4 | " " 12. |
| " | 10 | 10.70 | 51.3 | 1440 | 31.3 | 450 | 6.78 | 5.3 | " " 12. |
| " | 11 | 8.34 | | 1400 | 39.0 | 546 | 7.37 | 5.4 | " " 12. |
| " | 12 | 7.53 | | 1050 | 53.0 | 568 | 7.19 | 5.2 | " " 6. |
| " | 13 | 8.33 | 60.8 | 490† | 91.0 | 446 | 6.13 | 5.9 | Basal metabolism + 21 per cent. |
| " | 14 | 6.77 | 67.3 | 1760 | 51.2 | 678 | 10.40 | 5.6 | |
| 75 gm. glucose. | 1 | 6.25 | 60.3 | 1240 | 50.7 | 629 | 7.21 | 5.8 | Basal metabolism + 21 per cent. |
| Purine-free. | 1 | 6.15 | 64.7 | 850 | 83.2 | 715 | | 5.8 | |
| " | 2 | 4.50 | 69.5 | 1200 | 48.1 | 577 | 7.89 | 5.5 | |
| " | 3 | 3.34 | | 1420 | 53.2 | 755 | 6.82 | 5.8 | |
| " | 4 | 2.94 | 65.0 | 1810 | 24.9 | 452 | 6.73 | | |

* Urine for 18 hrs.

† Urine for 16 hrs.

*Effect of Variations in Water Balance.*

Laporte and Rouzaud (39) report that attacks of gout are accompanied by high plasma uric acid and hydremia, as measured by low viscosity of the blood. This is the opposite of the condition in fasting, in which anhydremia is associated with high plasma uric acid and the hydremia of the postfasting period with abnormally low concentration of uric acid in plasma. Harding and his associates (30), in their discussion of the hyperuricacidemia associated with submaintainance, high fat diets say, "The simplest explanation would be decrease in blood volume, leading to decreased capillary flow and hence decreased elimination through the kidneys." This explanation is open to various objections. Though diuresis accompanies fat diets and fasting, the actual reduction in blood volume may not be great. Gamble, Ross, and Tisdall (40) found that only 22 per cent of the loss in body water in the first days of fasting was extracellular water. Again, the diuresis which accompanies loss of body water presumably indicates not decreased but increased circulation through the capillaries of the kidneys. Also, retention of uric acid is not, so far as we are aware, present in conditions in which there is reduction in blood volume, as cholera and acute hemorrhage.

Finally, there is direct evidence against the view of Harding and associates in the experiment detailed in Table XIX and Fig. 11. Gamble and his associates (41) and J. B. S. Haldane (42) have shown that marked acidosis and diuresis follow the ingestion of acid-producing salts. The ingestion of 10 to 15 gm. of $CaCl_2$ a day by the subject L. R. produced loss of body water comparable to the loss accompanying fasting. Evidence of this was the marked negative water balance and the loss of 3.5 kilos in weight in 5 days, in spite of daily consumption of nearly 3000 calories of food. During fasting the subject's blood uric acid rose to 15 mg. per 100 cc. of blood, whereas during the period of $CaCl_2$ feeding, it did not exceed 4 mg. Another subject, while fasting, took 35 gm. of $CaCl_2$ during 2 days. Instead of increasing the degree of retention, there was evidence of slight release. These two experiments demonstrate that reduction in the volume of body water is not the sole cause of the uric acid retention of fasting. It is also difficult to make Harding's theory fit the facts in

TABLE XIX.

*Retention of Uric Acid during Fasting with Reference to the Effect of Atophan, Alkali, and of Acidosis Induced by Calcium Chloride.*

Epileptic subject. L. R., age 18, weight, 72.6 kilos.

| Diet. | Day. | Blood. | | | Urine. | | | Weight. | Remarks. |
|---|---|---|---|---|---|---|---|---|---|
| | | Uric acid per 100 cc. | | | | Uric acid. | | | |
| | | Blood. | Plasma. | Plasma bicarbonate. | Volume. | Per 100 cc. | Total output. | | |
| | | *mg.* | *mg.* | *vol. per cent $CO_2$* | *cc.* | *mg.* | *mg.* | *kg.* | |
| Purine-poor. | 1 | 4.5 | | | 680 | 82 | 561 | | |
| Fast. | 1 | 4.8 | | 65.6 | 555 | 52 | 289 | 72.6 | |
| " | 2 | 5.7 | | 53.7 | 1855 | 6 | 118 | 71.5 | |
| " | 3 | 6.8 | | 43.9 | 1110 | 7 | 78 | 69.0 | |
| " | 4 | 9.2 | | 47.6 | 1225 | 10 | 120 | 68.5 | |
| " | 5 | 9.1 | | 48.5 | 1400 | 10 | 140 | 67.7 | |
| " | 6 | 10.0 | | 46.5 | | | | | |
| | | 8.9 | 12.3 | 48.0 | 1575 | 21 | 327 | 67.5 | Atophan 1.5 gm. after blood was taken. |
| " | 7 | 9.4 | 11.8 | 45.9 | 1220 | 10 | 127 | 67.0 | |
| " | 8 | 9.7 | 11.5 | 44.9 | 1260 | 27 | 345 | 66.8 | Atophan 1.5 gm. $NH_4Cl$ 5.0 gm. |
| " | 9 | 15.0 | 16.0 | 44.5 | 845 | 39 | 331 | 66.3 | |
| " | 10 | 9.6 | 11.4 | 48.0 | | | | | |
| | | 11.6 | 22.5 | 55.8 | 965 | 20 | 189 | 67.2 | Alkali 125 gm. after blood was taken and during 2 days. |
| " | 11 | 8.1 | 8.8 | 76.6 | 1065 | 27 | 286 | 66.8 | |
| " | 12 | 8.3 | 11.4 | 66.4 | 955 | 23 | 222 | 66.0 | |
| " | 13 | 10.8 | 15.7 | 58.6 | 2070 | 13 | 275 | 65.8 | $CaCl_2$ 5 gm. |
| " | 14 | 11.2 | 13.9 | 54.5 | 1615 | 10 | 160 | 65.2 | |
| " | 15 | 8.2 | 15.4 | 48.3 | | | | | |
| | | 9.5 | 11.8 | 53.2 | 1405 | 50 | 707 | 65.2 | Atophan 5 gm. after first blood was taken (1 gm. every 2 hrs.). |
| High fat.* | 1 | 6.7 | 7.4 | 47.0 | 820 | 75 | 615 | 64.9 | |
| " " | 2 | 6.0 | 8.2 | 42.9 | 760 | 47 | 354 | 64.5 | |
| " " | 3 | 5.7 | 7.5 | 43.5 | 520 | 83 | 431 | 64.9 | |
| " " | 4 | 5.6 | 8.9 | 52.0 | 510 | 145 | 739 | 65.5 | |
| " " | 5 | 5.6 | 7.6 | 60.5 | 1800 | 39 | 394 | 66.0 | |

TABLE XIX—*Concluded.*

| Diet. | Day. | Blood. | | | Urine. | | | Weight. | Remarks. |
|---|---|---|---|---|---|---|---|---|---|
| | | Uric acid per 100 cc. | | Plasma bicarbonate. | Volume. | Uric acid. | | | |
| | | Blood. | Plasma. | | | Per 100 cc. | Total output. | | |
| | | *mg.* | *mg.* | *vol. per cent $CO_2$* | *cc.* | *mg.* | *mg.* | *kg.* | |
| Purine-poor.† | 1 | | | | 1690 | 38 | 651 | 65.6 | |
| " | 2 | 3.7 | | 69.0 | 645 | 115 | 741 | 66.0 | $CaCl_2$ 10 gm. |
| " | 3 | 3.5 | 4.7 | 68.5 | 670 | 126 | 844 | 64.2 | " 15 " |
| " | 4 | 3.2 | | 51.8 | 2565 | 27 | 685 | 65.5 | " 15 " |
| " | 5 | 3.8 | 4.8 | 48.5 | 2125 | 23 | 497 | 63.9 | " 15 " |
| " | 6 | 3.8 | 4.7 | | | | | | |
| | | 3.9 | | 40.9 | 1315 | 35 | 463 | 63.5 | " 15 " |
| " | 7 | 4.0 | 5.4 | 35.2 | 1080 | 42 | 454 | 63.5 | " 10 " |
| "‡ | 8 | | | | 1130 | 48 | 531 | 64.0 | " 10 " |
| " | 9 | 3.6 | 4.2 | 44.5 | 1280 | 43 | 546 | 64.5 | " 10 " |
| " | 10 | 3.7 | 4.3 | 47.5 | 1770 | 32 | 564 | 64.2 | " 15 " |
| " | 11 | 3.8 | | 46.6 | 1490 | 21 | 320 | 65.2 | " 15 " |
| " | 12 | | | 48.5 | 1100 | 46 | 502 | 65.1 | |
| " | 13 | 3.2 | | 58.5 | 780 | 55 | 428 | 65.3 | |
| " | 14 | | | | 900 | 53 | 478 | 65.8 | |

* Average for 5 days. Carbohydrate 25 gm., protein 55 gm., fat 230 gm., calories 2400.

† Average for 7 days. Carbohydrate 78 gm., protein 89 gm., fat 241 gm., calories 2800.

‡ Average for 7 days. Carbohydrate 170 gm., protein 76 gm., fat 215 gm., calories 2900.

regard to the action of chlorides. Harding and his associates (43) suggest that the increased elimination of uric acid which follows administration of NaCl is due to associated *increase* in blood volume. Yet the use of $CaCl_2$, which also causes increased elimination (Morris and Rees, 44), produces *decrease* in blood volume. In the case of subject L. R. (Table XIX) it will be noted that although he received $CaCl_2$ for 10 days, uric acid output was increased during the first 3 days, only. Calcium chloride, like carbohydrate, protein, and atophan, caused lowering of the level of uric acid in the body. When the new low level was reached, increased elimination ceased.

*Effect of Variation in Acidity of Urine.*

Although as a rule, the excretion of uric acid decreased with increasing acidity of the urine (*e.g.*, as in Table IV and Fig. 3) it was evident that this was not a causal relationship. For example, making the urine alkaline by means of alkali did not affect the amount of uric acid eliminated (Table XX and Fig. 12).

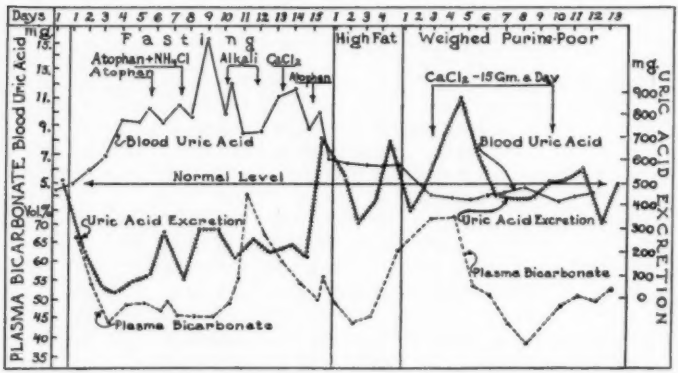


FIG. 11. (Illustrating part of Table XIX.) Fast of epileptic subject L. R. showing that fasting acidosis and reduction in blood volume was associated with uric acid retention whereas acidosis and reduced blood volume associated with ingestion of calcium chloride was not associated with uric acid retention.

*Effect of Acid-Base Changes in the Blood.*

Apparently little attention has been given to the statement of Umeda (27), made 10 years ago, that the decreased elimination of uric acid attending a fat diet was due to the accompanying ketosis. In the experiments by Harding *et al.* (30) and in those here recorded it is evident that the relationship of uric acid retention to ketosis is an important one.

The plasma bicarbonate was measured during twenty-one of the fasting periods. In those periods in which there were no complicating experimental procedures (as in Table I), the curves representing blood uric acid and plasma bicarbonate ran a reciprocal course, even to the subnormal blood uric acid and the

alkalosis which occurred together in the postfasting period. In general, subjects who were most active during fasting had the

TABLE XX.

*Effect of the Administration of Soda Bicarbonate on Uric Acid Retention.*

Healthy subject. W. L., age 38, weight 68 kilos.

| Blood. | | | | Urine. | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Per 100 cc. | | | | | Uric acid. | | | |
| Hr. taken. | Uric acid. | Non-protein N. | Plasma bicarbonate. | Period. | Volume. | Per 100 cc. | Total output. | Total N. | pH |
| | *mg.* | *mg.* | *vol. per cent $CO_2$* | | *cc.* | *mg.* | *mg.* | *gm.* | |
| Sept. 30 | | | | Sept. 30 | | | | | |
| 11.00 p.m. | 4.14 | 37.4 | 70.7 | 8–12 p.m. | 83 | 60 | 49 | 1.28 | 5.3 |
| Oct. 1 | | | | Oct. 1 | | | | | |
| | | | | 12– 4 a.m. | 168 | 27 | 45 | 1.24 | 5.3 |
| | | | | 4– 8 " | 202 | 37 | 76 | 1.42 | 6.2 |
| 10.00 a.m. | 3.91 | 36.8 | 72.5 | 8–12 " | 265 | 40 | 107 | 1.36 | 6.8 |
| 1.30 p.m. | 3.81 | 35.3 | 64.3 | 12– 4 p.m. | 150 | 40 | 61 | 1.26 | 5.3 |
| 6.00 " | 3.56 | 33.9 | 67.4 | 4– 8 " | 58 | 61 | 35 | 0.85 | 5.1 |
| 10.00 " | 4.32 | 34.3 | 60.0 | 8–12 " | 107 | 28 | 30 | 1.10 | 5.1 |
| Oct. 2 | | | | Oct. 2 | | | | | |
| | | | | 12– 4 a.m. | 162 | 12 | 20 | 1.01 | 5.0 |
| 4.15 a.m. | 4.30 | 34.3 | 59.6 | 4– 8 " | 113 | 19 | 22 | 0.84 | 5.0 |
| 10.00 " | 5.16 | 34.7 | 56.6 | 8–12 " | 137 | 18 | 24 | 1.03 | 5.0 |
| 2.00 p.m. | 5.10 | 35.0 | 52.2 | 12– 4 p.m. | 140 | 17 | 24 | 0.98 | 4.9 |
| 7.00 " | 5.33 | 36.4 | 53.9 | 4– 8 " | 186 | 13 | 25 | 1.55 | 4.7 |
| | | | | 8–12 " | 240 | 8 | 19 | 1.50 | 4.7 |
| Oct. 3 | | | | Oct. 3 | | | | | |
| 12.15 a.m. | 5.44 | 35.3 | 48.6 | 12– 4 a.m. | 263 | 7 | 19 | 1.64 | 4.6 |
| 7.30 " | 6.59 | 37.4 | 52.4 | 4– 8 " | 195 | 9 | 17 | 1.44 | 4.6 |
| 10.00 " | 6.61 | 34.5 | 45.2 | 8–12 " | 183 | 13 | 23 | | 4.7 |
| 11.55* " | 7.00 | 33.4 | 43.3 | | | | | | |
| 2.00 p.m. | 5.52 | 34.9 | 60.8 | 12– 4 p.m. | 180 | 14 | 25 | 1.52 | 4.9 |
| 4.30 " | 7.62 | 33.2 | 64.0 | 4– 8 " | 242 | 13 | 32 | 1.60 | 5.5 |
| 9.30 " | 7.77 | 33.7 | 65.5 | 8–12 " | 285 | 13 | 37 | 1.73 | 5.6 |
| Oct. 4 | | | | Oct. 4 | | | | | |
| 3.00 a.m. | 8.00 | 33.4 | 67.7 | 12– 4 a.m. | 320 | 9 | 28 | | 6.0 |
| | | | | 4– 8 " | 317 | 12 | 39 | 1.70 | 6.2 |
| 9.15 " | 8.62 | 30.8 | 69.5 | 8–12 " | 503 | 13 | 66 | 2.29 | 6.3 |
| 11.55† " | 8.25 | 33.4 | 68.3 | | | | | | |

TABLE XX—*Concluded.*

| Blood. | | | | Urine. | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Per 100 cc. | | | | | Uric acid. | | | |
| Hr. taken. | Uric acid. | Non-protein N. | Plasma bicarbonate. | Period. | Volume. | Per 100 cc. | Total output. | Total N. | pH |
| | *mg.* | *mg.* | *vol. per cent $CO_2$* | | *cc.* | *mg.* | *mg.* | *gm.* | |
| Oct. 4 | | | | Oct. 4 | | | | | |
| 2.30 p.m. | 8.42 | 32.1 | 76.5 | 12– 4 p.m. | 130 | 54 | 70 | 1.11 | 5.6 |
| | | | | 4– 8 " | 75 | 150 | 120 | 1.14 | 6.0 |
| 9.30 " | 5.88 | 35.8 | 77.3 | 8–10.30 p.m. | 50 | 250 | 125 | 0.75 | |
| Oct. 5 | | | | Oct. 5 | | | | | |
| | | | | 10.30 p.m.–6 a.m. | 155 | 142 | 221 | 2.14 | 5.6 |
| | | | | 6– 8 a.m. | 60 | 150 | 90 | 0.66 | 6.1 |
| 10.30 a.m. | 4.85 | 31.6 | 78.7 | 8–12 " | 135 | 137 | 185 | 1.11 | 6.0 |
| 12.45 p.m. | 4.21 | 30.4 | 74.3 | | | | | | |

* 12 o'clock noon, soda bicarbonate begun, 5 gm. every hr. for 6 hrs., then 10 gm. every 4 hrs. 75 gm. taken in the 24 hr. period.

† Mixed, purine-free food eaten at 12 noon, 6 p.m., 9 p.m., 8 a.m. Total food intake during the 24 hrs.: protein 81 gm., fat 93 gm., carbohydrate 209 gm., total calories 2052. 200 cc. of water were drunk every 4 hours throughout the experiment.

lowest plasma bicarbonate and the highest blood uric acid values. This reciprocal relationship, however, was only a general one. On various occasions the uric acid of the blood would vary, without coincident variation in the bicarbonate of the plasma. That ketosis and not simply acidosis was the factor of importance was suggested by the experiment mentioned above (Table XIX, Fig. 11). A degree of acidosis comparable to that accompanying fasting, was produced by the administration of $CaCl_2$. No retention of uric acid accompanied the resulting inorganic acidosis.

In another experiment on the healthy subject W. L. (Table XX and Fig. 12) the effect of administration of soda bicarbonate was tried. 75 gm. were taken during the last 24 hours of fasting without affecting the retention. Macleod and Haskins (45) and Umeda (27) found increase of uric acid excretion following moderate amounts of soda bicarbonate. J. B. S. Haldane (46) has

shown that ingestion of large amounts of bicarbonate causes ketosis. Presumably, in the case of W. L. the ingestion of 75 gm. of bicarbonate increased the existing fasting ketosis. Therefore, the lack of any release of uric acid retention following ingestion of bicarbonate adds evidence to the importance of ketosis in uric acid retention.

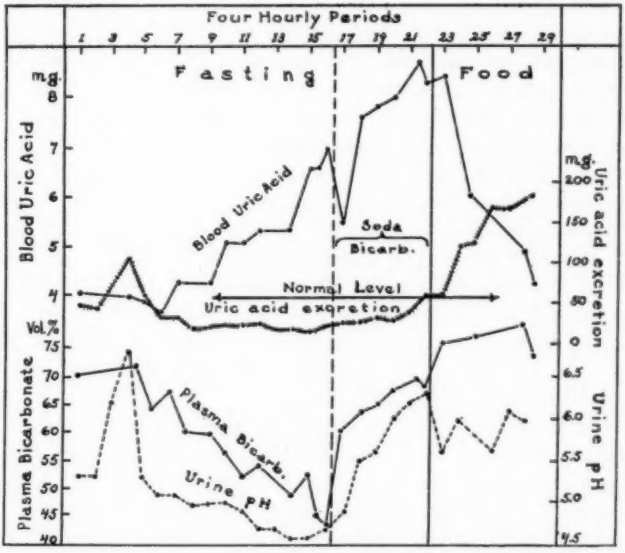


FIG. 12. (Illustrating Table XX.) 4 days fast of healthy subject W.L. with observations every 4 hours, showing that ingestion of 75 gm. of soda bicarbonate during last 24 hours did not influence the concentration of blood uric acid or the amount of uric acid excreted, though it increased plasma bicarbonate and decreased pH of the urine.

A proprietary mixture of various alkalies, containing also citrate and 2 per cent sodium chloride, was given in various amounts for from 2 to 4 days during six fasting periods (Table XIX shows one of these). In four of the periods, no effect on uric acid retention was apparent. In two, there was gradual and partial release of the retention. Possibly the apparent effect in these two cases was due to the sodium chloride. Certainly alkali had little effect in relieving uric acid retention.

DISCUSSION.

The large variations in uric acid excretion which attend fasting acidosis may have their counterpart in small variations associated with comparatively minor changes in acid-base relationships. For example, the well known diurnal and hourly fluctuations in uric acid elimination may be due, not to variations in production as is supposed, but to variations in the level of uric acid in blood and tissues associated with changes in alkalinity of body fluids consequent on digestion. 20 years ago, Folin (47) pointed out that a potato diet caused increased elimination of uric acid. Reference to his tables shows that on these potato days there was decrease in the total acidity of the urine. In the preceding tables we have seen that the release of the uric acid retention of fasting by feeding of carbohydrate or protein was attended by marked alterations of acid-base relationships in blood and urine. Whether the increased elimination of uric acid which follows change from a mixed to a high protein or high carbohydrate diet is attended by measurable acid-base changes is a matter for investigation. The lack of measurable change would not be conclusive for, as Rous (48) has recently shown, changes in the pH of tissues may not parallel changes of pH in blood.

The important question arises whether the responsibility for uric acid retention rests with the kidneys or with the body tissues. The kidneys have assumed a new prominence since Folin and his associates (15) found that an undue proportion of injected uric acid is held by them. It is clear that the kidneys during fasting are able to concentrate uric acid. It seems evident, however, that the kidneys with their variable threshold for uric acid are the immediate factors in its retention and release. If we accept for fasting retention the explanation advanced by Folin (15) and his associates for gout, we conclude that the kidneys do not absorb circulating uric acid to the normal extent. But even with this considerable advance in our knowledge the question yet remains, What prevents the kidneys from absorbing uric acid? Is the disability in the kidneys primary, or only secondary to metabolic change elsewhere in the body? The fact that fasting retention is temporary and coexistant with severe metabolic disturbances in the body tissues, that its development is a process consuming days, that its release requires relatively large amounts

of carbohydrate or protein food, and that the first step in this release by small quantities of food is increased elimination without lowering of the renal threshold, all these facts speak for a process which involves the whole body. It seems evident that kidneys and general body tissues both play a part in fasting retention. It would seem probable that the same is true for gout.

We have examined a number of factors affecting retention, none of which standing alone seems sufficient to explain the phenomenon. An immediate objection to any relationship between ketosis and uric acid retention is the fact that retention does not accompany the ketosis of diabetes. There is, however, an important difference between the acidosis of fasting and of diabetes. The former is associated with hypoglycemia and the latter with hyperglycemia. Rémond and Rouzaud (32) have shown that the blood of patients with gout and diabetes has a lower uric acid content than blood of patients with gout alone, and that intravenous injection of glucose lowers blood uric acid. On the other hand, non-diabetic ketosis, strangely enough, is not constantly associated with hypoglycemia and ready utilization of glucose. Weeks *et al.* (49) have found that high blood sugar accompanies a fat diet very high in calories. Sevringhaus (50) has observed, as we have also, that ingestion of glucose during fasting results in an unduly prolonged hyperglycemia.

Disturbance in oxidation and variations in pH are intimately related. Thus Koehler and Reitzel (51) find that oxygen consumption of tissues diminishes with variations in their pH from normal, and Rous (48) observes that the pH of various body tissues may differ greatly, and may become more acid with local circulatory disturbances. (Incidentally, in view of the dependence of solubility of uric acid on the pH of the solution, the fact that certain tissues, *e.g.* connective tissue and tendons, are much more alkaline than others, may have influence on the characteristic location of nodules in gout.) Chlorides, also, may play a part, as suggested by the fact that their ingestion increases uric acid elimination, and chlorine almost disappears from the urine during fasting.

Of the series of physiological and chemical changes in body structure and function which occur during fasting, disturbance in the three following might be concerned in uric acid retention:

(1) equilibrium of electrolytes (with resulting changes in water balance); (2) acid-base relationships of body tissues, including kidney, or blood; (3) oxidation of fat with associated utilization of glucose. Because variations in acid-base relationships are more easily measured, they may have received undue prominence in this study. Disturbances in oxidation may be more fundamental. Some writers have explained the high blood uric acid found in cases of severe anoxemia, *e.g.* carbon monoxide poisoning, on the basis of diminished destruction (oxidation) of uric acid. Though that theory is discredited, it is possible that decreased or incomplete oxidation of body material results, not in decreased oxidation of uric acid, but in its retention.

All the three possible factors which have been mentioned are interrelated. Their proper correlation, and the exact mechanism involved in retention of uric acid, must await other investigators, and new knowledge. The complete explanation, when it comes, will probably not be simple. The problem of uric acid retention in fasting is probably, in its essentials, the old, unsolved problem of gout.

## CONCLUSIONS.

Measurements of uric acid in blood and urine during 21 fasting periods revealed the following facts.

1. The observed high concentrations of uric acid in blood were due to decrease in uric acid excretion. The usual diurnal and hourly variations in excretion were abolished. Although concentration of uric acid in urine was very low (sometimes less than in blood plasma), the kidneys were able to concentrate uric acid to a normal extent. Decreased urine volumes seemed to be associated with increased uric acid output.

2. Study of quantitative relationships (mainly from a healthy subject) showed that retention was evident first in plasma; with rising concentrations in the plasma an increasing proportion of retained uric acid (up to about 80 per cent) was found within cells; at the higher levels there was apparent equilibrium between concentration within and without the cells; the increase of concentration in tissue water, at its peak, was 30 per cent of the increase in plasma; and approximately 60 per cent of uric acid retained during fasting was recovered in the postfasting period, the unrecovered

fraction presumably being either retained in the tissues or destroyed. During some of the 24 hour periods there was considerable shifting of uric acid from blood to tissues and *vice versa*.

3. During fasting a smaller proportion than normal of uric acid injected intravenously was recovered in the urine.

4. Feeding of fat did not relieve retention, whereas, feeding of carbohydrate, thyroid extract, amino acids, and protein did. Protein (one experiment) caused a more prolonged increased elimination than amino acid and glucose. Following ingestion of small amounts of food, there was increased elimination of uric acid without lowering of the renal threshold.

5. Following ingestion of atophan, in doses of 4 to 5 gm., there was lowering of the renal threshold with drainage of uric acid first from the blood, and then from tissues.

6. From the foregoing data it seems evident that variations in excretion of uric acid following the use of various purine-free diets do not mean variations in production, but variations in the amounts of uric acid contained in blood and tissues. The observations suggest the use of a low fat diet in the treatment of gout.

7. Disturbance in kidney function and in metabolism of the body both played a part in the retention. Of various possible factors examined, the attending ketosis seemed the most constant. It is probable that various interrelated disturbances in oxidation and in the acid-base equilibrium of the blood and body tissues are involved.

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# THE EFFECT OF VITAMIN A DEFICIENCY UPON THE CHARACTER OF NITROGEN METABOLISM.

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In spite of much detailed work upon the effects of vitamin A deficiency a tenable theory of the mode of action of this food accessory has not yet been brought forward. McCarrison (1) has suggested that a disturbance in the permeability of the intestinal wall may occur in avitaminosis and result in increased absorption of toxins. Thus the symptoms of vitamin deficiency may result from toxemias due to the lack of ability of the organism to synthesize substances protecting against these toxins. Dragstedt and Cooper (2) tested this theory by joining rats in pairs in parabiotic style so that the abdominal cavity was common to both. One of the pair was then fed an excess of vitamin A while the other was deprived of this accessory. The results were the same as though the animals had been separate. This seems to show that no toxemia could have been involved in the vitamin A deficiency, otherwise both animals would have developed symptoms.

Another theory often suggested is that the vitamins stimulate metabolic processes. But excess feeding of vitamins produces no overstimulation, as is the case with thyroid extract for example. No effect of avitaminosis upon basal metabolism has so far been observed which cannot be attributed to accompanying lowered food intake and decrease in body substance.

Defense against bacterial invasion has also been suggested as one of the functions of vitamin A. The frequent cases of pus formation in nasal passages, middle ear, mastoid cells, and lungs observed by Daniels and Armstrong (3) in rats, and Beach (4) in chickens suffering from vitamin A deficiency appear to support this view.

Some years ago Tschirch (5) suggested that one function of the vitamins might be that of ring-closing catalysts for the formation of purines. It is well known that large amounts of purine-containing nuclear materials may be formed by growing animals upon practically purine-free diets. Tschirch put forward the suggestion that the purine nucleus may be formed from certain non-cyclic amino acids through the intervention of one or another of the vitamins, enzyme-like substances which are closely related to or contain vitamins. He advanced no experimental evidence in support of this theory, and confined it particularly to the vitamin B deficiency syndrome as seen in beriberi.

The experiments of Ackroyd and Hopkins (6) opened a new field of speculation as to purine synthesis in the animal body. They found that on diets free from arginine and histidine young rats ceased to grow, and at the same time excreted diminished amounts of allantoin. Ackroyd (7) had previously shown that allantoin is the chief end-product of purine catabolism in the rat as in most other mammals. The excretion of allantoin was found to be about fifteen times greater than that of uric acid and free purines. Folin and Morris (8) after a more or less complete study of the nitrogen partition in rat urine came to the conclusion that rats exhibited a high uric acid output similar to that of man rather than to that of other mammals. They made no separate allantoin determinations but unintentionally apparently included this nitrogen in their figure for urea. Hunter, Givens, and Guion (9) using the careful technique of Wiechowski for allantoin determinations determined the "uricolytic index" in rats as well as in many other animals. They found no reason to assume that any lower degree of uricolysis exists in rats than in other mammals, and no resemblance to man in this respect. They concluded that the relatively large output of uric acid observed by Folin and Morris was merely an outcome of the high level at which the purine as well as the total protein metabolism of the rat is pitched.

In the experiments of Ackroyd and Hopkins (6) the removal of arginine and histidine from the diet of young rats caused a decrease of 40 to 50 per cent in allantoin output. The restoration of either or both of these amino acids was followed by resumption of growth and increase in allantoin excretion. Rose and Cox (10) repeated this work with some changes in procedure and found that when histidine was restored to the diet of young rats which had ceased to grow on the arginine-histidine-free diet growth was resumed, but that this was not the case when arginine was added. Apparently these two amino acids are not interchangeable in this function. Rose and Cook (11) supplemented this study by allantoin and uric acid determinations on rats fed all these diets, and found that again the allantoin output was diminished in the absence of histidine, although not much affected by the presence or absence of arginine. Cod liver oil was included in the diets described in both these latter papers in amounts adequate to provide the fat-soluble vitamins necessary.

Several studies by German investigators have recently appeared describing the changes in nitrogen metabolism which accompany complete avitaminosis. Bickel (12), Adachi (13), Hirabayashi (14), Collazo (15), Yushiue (16), all used dogs as subjects and diets lacking in all known vitamins. Adachi determined uric acid and allantoin excretion and found that both increased gradually until just before death. Collazo also found a slight rise in purine and uric acid excretion with fall before death. Bickel reports that both fat and protein absorption and oxidation are quantitatively elevated in avitaminosis, but are for the most part qualitatively normal. Carbohydrate metabolism is accomplished by less than normal oxygen consumption and carbon dioxide production, with resulting formation of toxic intermediary products. An elevated total metabolism accompanies

the depressed respiration, and the incomplete oxidation of protein and fat is over-balanced by the incomplete oxidation of sugar.

Yushiue made determinations in vitamin-starved dogs of the excretion of urea, ammonia, uric acid, allantoin, and amino acids over long periods during which the nitrogen balance was also observed. He found that disturbances of absorption in the alimentary canal were accompanied by an increase in urea and a decrease in ammonia and amino acid nitrogen in the urine. As the diseased condition advanced the nitrogen balance became more and more negative. Even forced feeding of large amounts of protein failed to produce a positive balance. Apparently then the increased breakdown of body protein although not qualitatively abnormal is a primary factor in vitamin starvation. The cells take up body protein readily, but immediately burn it, but the burning is apparently along the usual path.

Bickel (17) has summarized other work of a similar kind carried on in his laboratory, showing that only about one-fourth of the total loss in weight in avitaminosis is due to destruction of body protein, the remainder being due to burning of fat and carbohydrate. In spite of this implied increase in oxidation a lowered oxygen consumption was noted. The explanation offered is that the body cells under these conditions fail to utilize absorbed food materials, with consequent breakdown of the tissues.

Since none of these authors report differentiation among the various avitaminoses it appeared important and interesting to determine the nature of nitrogen excretion in growing rats fed normal and vitamin-deficient diets. Since there is no reason to suppose that all the vitamins function in the same manner such an inquiry should be made separately for each vitamin known to be required for the growth and well being of a given species. The results here reported are concerned only with vitamin A, but studies of a similar kind with vitamin B-deficient rats have also been made in this laboratory.

*Procedure.*

Only one diet was used in these experiments, the usual basal diet supposedly adequate in all respects except as to vitamin A. It was made up as follows:

| | |
|---|---|
| Casein | 18 |
| Agar | 2 |
| Salt mixture | 4 |
| Crisco | 20 |
| Dextrin | 56 |

The casein was rendered vitamin A-free by the method advocated by Coward and Drummond (18), that of heating at 110–

120°C. for 24 hours with frequent raking over to assist oxidation. This method has been in use for a number of years in this laboratory and has been found uniformly satisfactory both as to removal of vitamin A and lack of harmful effects upon the availability of the casein. The salt mixture used was that suggested by Osborne and Mendel (19). The dextrin was prepared from cornstarch by baking at 150–160°C. The make-up of the diet and care of the animals have been fully described elsewhere (20).

Dried yeast was supplied all the rats as source of vitamin B, 0.3 gm. daily being given separately from the rest of the food. Cod liver oil was used as source of vitamin A for the animals kept on complete diet, 25 mg. being the daily dose per rat.

In most cases two or three young rats were confined in the same metabolism cage in order to increase the amount of the total urinary collections. When adult animals were used these were caged individually.

The method of caging the animals and collecting urine and feces was developed in this laboratory independently, but is essentially the same as that described by Mitchell (21). The rats are confined in cylindrical wire netting cages resting on wire net screens of $\frac{1}{4}$ inch mesh, the screens in turn being supported upon glass plates 8 inches in diameter and about 1 inch in depth. In the bottom of the glass plates were placed daily fresh circles of filter paper which had been soaked in N/10 hydrochloric acid and dried before use in the cages. This was done to prevent loss of ammonia from the urine and to prevent the breakdown of allantoin, since it has been reported by Givens (22) that allantoin is stable in acid solution but unstable in neutral and alkaline solutions.

The experiments were usually of 3 days duration, but the collections of the urine were made daily. The animals and food were weighed only at the beginning and end of each period.

The urine sample was collected daily by removing all visible traces of food particles and feces from the filter paper and then washing it carefully and repeatedly with small portions of warm distilled water. The sides and bottom of the cage and pan were also rinsed to insure complete collection. All of these washings were collected in a glass jar and sufficient N/10 HCl was added to make the solution slightly acid to litmus. Toluene was added

as a preservative and the sample was placed in the refrigerator until the end of the period, when the analysis was carried out. In order to determine how completely the urine had been removed from the filter paper by this procedure, the washed papers were repeatedly analyzed for nitrogen but were found to be nitrogen-free.

The total urinary collection for the 3 day period, usually about 600 cc., was filtered and analyzed at the end of each period. Daily analyses for urea and uric acid were made in several cases and were checked with results of analyses of the 3 day sample, but this was found to present no advantage, so was discontinued.

The urine was analyzed for total nitrogen, urea, allantoin, uric acid, creatinine, and creatine.

*Total nitrogen* was determined by the Kjeldahl method, 20 cc. of urinary washings being used for each determination. Total nitrogen determinations were made in several cases both before and after attempted removal of protein by boiling with acetic acid according to the Scherer coagulation method. No differences were found, thus showing that no appreciable amount of protein from the basal diet was present.

*Allantoin* was determined by the method of Plimmer and Skelton (23). This is based upon the magnesium chloride method of Folin (24) for the determination of urea by its hydrolysis to ammonium carbonate, but in which allantoin hydrolysis has also been shown to take place. Since urease does not decompose allantoin and since both are quantitatively determined by Folin's magnesium chloride method, the amount of allantoin is the difference.

Since several modifications of the original method were made and since the description as given by the original authors and by Harding and Young (25) is not particularly detailed concerning some steps, the method is here again described.

50 cc. of urinary sample were used because of the dilution of the urinary washings obtained. This was placed in a 500 cc. Kjeldahl flask, along with 5 cc. of concentrated HCl, 20 gm. of crystalline $MgCl_2$, and a small cube of paraffin. The nitrogen content of these substances was determined and subtracted from the total allantoin-urea-$NH_3$ (Folin) nitrogen in each case. A reflux condenser was then attached to the Kjeldahl flask. A large thistle tube with an elbow of glass tubing at the top was fitted

to the top of the condenser to prevent spattering of the contents. Micro burners were used for the heating, during the initial part of which the condenser jacket was left empty. This initial boiling was continued for about 30 minutes until the mixture was concentrated to the point where marked and permanent frothing occurs. The flame was then turned low, and gentle boiling continued for 2 hours during which time water was run through the jacket of the condenser. The flasks were then allowed to cool in position and the thistle tube and condenser rinsed out with 50 cc. of distilled water. The contents of the flasks were then neutralized and ammonia was distilled off in the usual Kjeldahl fashion. The distillation is complicated by the excessive bumping caused by the viscous $Mg(OH)_2$ formed when excess NaOH is added. Care was taken to use only a slight excess of alkali in order to prevent this difficulty.

*Check upon Plimmer and Skelton Method for Allantoin.*

Allantoin was prepared from uric acid by oxidation with potassium permanganate, and the product was recrystallized several times from hot water. The nitrogen content of the allantoin crystals was found to be 21.11 per cent, as compared with the theoretical figure of 21.53 per cent.

10 cc. of 0.5 per cent solution of this product were added to the usual 20 cc. of rat urine sample and the allantoin content of the mixture was determined by the method described above. The figure thus obtained was compared with that yielded by the application of the method to another sample of the same urine alone. This check was made several times before confidence in the method was established.

The best and the poorest recoveries obtained in these checks are given below.

| | gm. |
|---|---|
| 1. N content of added allantoin | 0.0105 |
| " " " allantoin plus urine sample | 0.0148 |
| " " " urine sample | 0.0051 |
| Per cent recovery of added allantoin N, 92.3. | |
| 2. N content of added allantoin | 0.0105 |
| " " " allantoin plus urine sample | 0.0201 |
| " " " urine sample | 0.0099 |
| Per cent recovery of added allantoin N, 97.1. | |

*Urea* was determined by the urease method of Van Slyke and Cullen (26) as modified by Youngburg (27), except that ammonia was not removed by permutit powder, since this was also recovered in the Folin nitrogen of the allantoin determination. The aeration and subsequent titration method was used because it is more nearly parallel with the procedure used in the total nitrogen and allantoin titrations.

*Uric acid* was determined by the colorimetric method of Benedict and Franke (28). 20 cc. of sample were used in 100 cc. flasks, 5 cc. of NaCN solution and 1 cc. of arsenophosphotungstic acid were added, the blue color produced compared colorimetrically with a 0.1 mg. uric acid standard.

In some cases, a final washing of the filter papers with a saturated lithium carbonate solution was used to insure complete recovery of the uric acid. This, however, was not found to increase the amount of uric acid collected, and so was discontinued. Daily determinations of uric acid, as previously mentioned, were also made, and the total compared with the results of the single determination made at the end of the 3 day period. There was found to be no increase in uric acid detected by this modification.

*Creatinine* was determined by the sodium picrate method of Folin. 10 cc. of urine sample were used in 200 cc. volumetric flasks, and comparisons were made colorimetrically with 0.25 or 0.30 mg. pure creatinine standards.

*Creatine* determinations were made in all cases by the method of Benedict and Myers (29), but since the results were found to be unusually high and uneven they are not given here. This part of the work is being repeated with particular attention to the possible sugar content of the urines.

The formol titration of Henriques and Sörensen (30) for amino acids was carried out on two samples, but amino nitrogen was found to be practically negligible in amount. This determination was therefore not continued.

Analysis of the rat urine for purine bases was carried out in several samples according to Welker's modification of the method of Arnstein and Salkowski, but with practically negative results.

## DISCUSSION OF RESULTS.

The results of the determinations of urinary nitrogen partition in young growing rats fed the basal diet with additions of 0.3

TABLE I.

*Urinary Nitrogen Partition in Young Rats on Diets Adequate as to Vitamins A and B.*

| Rat No. | Period No. | Length of period. | Body weight. | Gain or loss in body weight. | Food eaten. | Total urinary N. | Allantoin N. | | Urea + $NH_3$ N. | | Creatinine. | | Uric acid. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *days* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *per cent total N* | *gm.* | *per cent total N* | *mg.* | *per cent total N* | *mg.* | *per cent total N* |
| 984, 988 | I | 3 | 149 | +3 | 37 | 0.268 | 0.016 | 6.07 | 0.108 | 40.3 | 18.93 | 2.62 | 4.99 | 0.62 |
| | II | 3 | 140 | −4 | 38 | 0.237 | 0.028 | 12.02 | 0.106 | 44.7 | 17.41 | 2.72 | 4.45 | 0.62 |
| | III | 3 | 148 | +4 | 39 | 0.244 | 0.013 | 5.28 | 0.091 | 37.4 | 20.29 | 3.09 | 3.81 | 0.52 |
| Average..... | | 3 | 145 | +1 | 38 | 0.250 | 0.019 | 7.79 | 0.102 | 40.8 | 18.88 | 2.81 | 4.42 | 0.59 |
| 984, 989 | I | 3 | 227 | ±0 | 43 | 0.213 | 0.031 | 14.6 | 0.107 | 50.0 | 21.17 | 3.70 | 3.42 | 0.53 |
| | II | 3 | 226 | +6 | 25 | 0.184 | 0.023 | 12.4 | 0.079 | 42.6 | 17.35 | 3.49 | 3.05 | 0.55 |
| | III | 3 | 226 | +2 | 25 | 0.177 | 0.027 | 14.5 | 0.088 | 43.2 | 23.61 | 4.90 | 3.29 | 0.62 |
| Average..... | | 3 | 226 | +2.7 | 31 | 0.191 | 0.027 | 13.8 | 0.091 | 45.3 | 20.71 | 4.03 | 3.25 | 0.57 |
| 988, 989 | I | 3 | 197 | +24 | 44 | 0.467 | 0.030 | 6.3 | 0.191 | 41.0 | 19.03 | 1.52 | | |
| | II | 3 | 195 | −2 | 21 | 0.376 | 0.045 | 11.9 | 0.173 | 45.9 | 22.13 | 2.19 | | |
| | III | 3 | 202 | +7 | 33 | 0.405 | 0.034 | 8.3 | 0.171 | 42.3 | 18.60 | 1.71 | | |
| Average..... | | 3 | 198 | +9.7 | 33 | 0.416 | 0.036 | 8.8 | 0.178 | 43.1 | 19.93 | 1.81 | | |
| Young rats, adequate vitamin A but no yeast in diet. | | | | | | | | | | | | | | |
| 988, 989 | IV | 2 | 210 | +8 | 27 | 0.240 | 0.019 | 8.0 | 0.105 | 43.5 | 11.20 | 1.74 | | |

TABLE II.

*Urinary Nitrogen Partition in Young Rats on Vitamin A-Deficient, but Otherwise Adequate Diet.*

| Rat No. | Period No. | Length of period. | Body weight. | Gain or loss in body weight. | Food eaten. | Total urinary N. | Allantoin N. | | Urea + $NH_3$ N. | | Creatinine. | | Uric acid. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *days* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *per cent total N* | *gm.* | *per cent total N* | *mg.* | *per cent total N* | *mg.* | *per cent total N* |
| 982, 985, 986, 987 | I | 3 | 361 | −12 | 68 | 0.404 | 0.016 | 3.9 | 0.151 | 37.2 | 33.22 | 3.05 | 12.76 | 0.99 |
| | II | 3 | 370 | −6 | 49 | 0.309 | 0.033 | 10.0 | 0.104 | 34.2 | 27.46 | 3.29 | 6.84 | 0.74 |
| | III | 3 | 381 | −1 | 85 | 0.509 | 0.066 | 13.0 | 0.175 | 34.4 | 37.30 | 2.52 | 7.52 | 0.49 |
| Average..... | | 3 | 371 | −6.3 | 67 | 0.407 | 0.038 | 9.0 | 0.143 | 35.3 | 32.66 | 2.95 | 9.04 | 0.74 |
| 992, 993, 994 | I | 3 | 149 | −8 | 36 | 0.246 | 0.015 | 6.1 | 0.095 | 37.5 | 19.26 | 2.91 | 6.44 | 0.87 |
| | II | 3 | 151 | +3 | 38 | 0.253 | 0.031 | 12.2 | 0.106 | 42.0 | 17.98 | 2.64 | 5.92 | 0.78 |
| | III | 3 | 155 | −2 | 44 | 0.270 | 0.025 | 9.4 | 0.099 | 36.7 | 19.86 | 2.73 | 3.98 | 0.49 |
| Average..... | | 3 | 152 | −2.3 | 39 | 0.256 | 0.024 | 9.2 | 0.100 | 38.7 | 19.03 | 2.76 | 5.45 | 0.71 |
| 990, 991 | I | 3 | 113 | −3 | 24 | 0.369 | 0.028 | 7.63 | 0.153 | 41.3 | 16.44 | 1.62 | | |
| | II | 3 | 105 | −8 | 23 | 0.465 | 0.035 | 7.61 | 0.190 | 40.8 | 13.50 | 1.08 | | |
| | III | 3 | 96 | −9 | 17 | 0.299 | 0.011 | 3.60 | 0.123 | 41.1 | 12.21 | 1.51 | | |
| Average..... | | 3 | 105 | −6.6 | 21 | 0.378 | 0.025 | 6.28 | 0.155 | 41.1 | 14.05 | 1.40 | | |
| Young rats, diet deficient in both vitamins A and B. | | | | | | | | | | | | | | |
| 990, 991 | IV | 2 | 84 | −12 (Both died.) | 1 | 0.200 | 0.030 | 15.10 | 0.103 | 51.5 | 5.86 | 1.09 | | |

TABLE III.

*Urinary Nitrogen Partition in Adult Rats on Diets Adequate as to Vitamins A and B.*

| Rat No. | Period No. | Length of period. | Body weight. | Gain or loss in body weight. | Food eaten. | Total urinary N. | Allantoin N. | | Urea + $NH_3$ N. | | Creatinine. | | Uric acid. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *days* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *per cent total N* | *gm.* | *per cent total N* | *mg.* | *per cent total N* | *mg.* | *per cent total N* |
| F 8 | I | 2 | 228 | −5 | 22 | 0.235 | 0.044 | 18.9 | 0.113 | 48.2 | 16.73 | 2.67 | 3.59 | 0.51 |
| | II | 2 | 225 | −3 | 19 | 0.188 | 0.017 | 8.8 | 0.097 | 51.6 | 16.93 | 3.35 | 2.86 | 0.50 |
| | III | 2 | 221 | +5 | 34 | 0.144 | 0.006 | 4.2 | 0.061 | 42.3 | 15.17 | 3.94 | 3.83 | 0.89 |
| Average..... | | 2 | 225 | −1 | 25 | 0.189 | 0.022 | 10.6 | 0.090 | 47.4 | 16.28 | 3.32 | 3.43 | 0.63 |
| F 26 | I | 2 | 202 | −1 | 22 | 0.235 | 0.059 | 25.1 | 0.122 | 52.1 | 16.90 | 2.67 | 2.71 | 0.38 |
| | II | 2 | 209 | +7 | 26 | 0.195 | 0.044 | 22.6 | 0.095 | 48.7 | 17.00 | 3.24 | 2.48 | 0.42 |
| | III | 2 | 203 | +10 | 19 | 0.117 | 0.005 | 4.6 | 0.057 | 48.8 | 15.24 | 4.84 | 3.41 | 0.97 |
| Average..... | | 2 | 205 | +5.3 | 22 | 0.182 | 0.036 | 17.4 | 0.091 | 49.9 | 16.38 | 3.58 | 2.87 | 0.59 |

TABLE IV.

*Average Urinary Nitrogen Partition of Rats on Diets Adequate and Deficient in Vitamin A, Calculated per Kilo of Body Weight.*

| No. of rats. | No. of 3 day periods. | Food eaten. | Gain or loss in body weight. | Total urinary N. | Allantoin + urea + $NH_3$ N. | | Allantoin N. | | Urea + ammonia N. | | Creatinine. | | | Uric acid. | | Remarks. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Per cent of total N. | | Per cent of total N. | | Per cent of total N. | | N | Per cent of total N. | | Per cent of total N. | |
| **Young rats, diet adequate in vitamins A and B.** | | | | | | | | | | | | | | | | |
| | | *gm.* | *gm.* | *gm.* | *gm.* | | *gm.* | | *gm.* | | *mg.* | *mg.* | | *mg.* | | |
| 2 | 3 | 261 | +6.2 | 1.72 | 0.83 | 48.6 | 0.133 | 7.79 | 0.701 | 40.8 | 130.17 | 48.4 | 2.81 | 30.30 | 0.59 | All animals normal. |
| 2 | 3 | 143 | +38.3 | 0.84 | 0.51 | 59.1 | 0.118 | 13.80 | 0.403 | 45.3 | 91.47 | 34.0 | 4.00 | 14.38 | 0.57 | |
| 2 | 3 | 165 | +42.1 | 2.10 | 1.08 | 51.9 | 0.182 | 8.85 | 0.902 | 43.1 | 100.70 | 37.4 | 1.81 | | | |
| Average.... | | 190 | +28.8 | 1.55 | 0.81 | 53.2 | 0.144 | 10.14 | 0.669 | 43.1 | 107.44 | 39.9 | 2.87 | 22.34 | 0.58 | |
| **Young rats, diet deficient in vitamin A.** | | | | | | | | | | | | | | | | |
| 4 | 3 | 183 | −17.3 | 1.10 | 0.49 | 44.2 | 0.101 | 9.00 | 0.388 | 35.3 | 88.04 | 32.04 | 2.95 | 24.52 | 0.76 | All rats show well marked symptoms of vitamin A deficiency. |
| 3 | 3 | 259 | −15.6 | 1.69 | 0.82 | 48.3 | 0.161 | 9.25 | 0.654 | 38.7 | 125.49 | 46.7 | 2.76 | 36.02 | 0.71 | |
| 2 | 3 | 203 | −65.5 | 3.60 | 1.71 | 47.3 | 0.233 | 6.68 | 1.48 | 41.1 | 133.80 | 49.7 | 1.40 | | | |
| Average.... | | 215 | −32.8 | 2.13 | 1.01 | 46.6 | 0.165 | 8.31 | 0.841 | 38.4 | 115.77 | 42.8 | 2.37 | 30.27 | 0.73 | |
| **Adult rats, diet adequate in vitamins A and B. (Averages for 2 day periods.)** | | | | | | | | | | | | | | | | |
| 1 | 3 | 113 | −4.2 | 0.84 | 0.50 | 58.0 | 0.099 | 10.63 | 0.415 | 47.4 | 72.4 | 26.9 | 3.32 | 15.25 | 0.63 | |
| 1 | 3 | 109 | +35.9 | 0.89 | 0.62 | 67.3 | 0.175 | 17.41 | 0.44 | 49.7 | 80.9 | 30.0 | 3.58 | 14.2 | 0.59 | Normal. |
| Average.... | | 111 | +15.8 | 0.86 | 0.56 | 62.7 | 0.137 | 14.02 | 0.428 | 48.5 | 76.6 | 28.4 | 3.45 | 14.72 | 0.61 | |

gm. of dried brewers' yeast and 0.025 gm. of cod liver oil as sources of vitamin B and vitamin A respectively are given in Table I. One period of 2 days is included during which yeast was withheld, as a check upon the allantoin production due to the nucleic acid of the yeast. It will be noted that no definite difference in allantoin excretion is ascribable to the small amount of yeast used. In Table II similar data are presented for young rats fed only the yeast in addition to the basal diet, and in Table III for adult rats receiving both yeast and cod liver oil. Table IV presents a recalculation of all averages in terms of excretion per kilo of body weight of the animals.

*Allantoin, Urea, and Ammonia Nitrogen.*

These three substances were determined together by the $MgCl_2$ method of Plimmer and Skelton (23) modified as described previously. The peculiarity of every determination made in this study was the low percentage of recovery, ranging for adults from 77.2 to 46.5 per cent of total urinary nitrogen. In the case of the young rats on a diet adequate in vitamin A the per cent ranged from 64.6 to 42.7, while those on a diet inadequate in vitamin A ranged from 54.6 to 41.1 per cent. To this amount of nitrogen may be added roughly 6 per cent from creatinine, creatine, and uric acid. This still leaves a high percentage of undetermined nitrogen, which must occur in some form not tested for in this study. Similar work, as yet unreported, done in this laboratory by other workers, has resulted in the same range of recovery.

In adult rats, fed upon a diet adequate in vitamin A, the per cent of total nitrogen represented by allantoin, urea, and ammonia is higher than it is for young growing rats also fed a diet adequate in vitamin A. In all cases, whether adult or growing rat fed upon an adequate diet, the percentages of allantoin-urea-ammonia nitrogen decrease directly with the gain in weight. In the young growing rats, the same relationship holds true. Without exception, for all animals upon a diet adequate in vitamin A, the per cent of nitrogen represented by allantoin-urea-ammonia is highest during the period of greatest loss in body weight (or least gain) and the periods of greatest gain in weight are correspondingly those of the least per cent of excretion of nitrogen in these forms. The same relationship holds true for the excretion per kilo of body weight.

In the case of young animals suffering from a lack of vitamin A, an entirely different picture is presented. The exact reverse of the conditions found for animals upon an adequate diet is shown. In every case, the greatest gain in weight or least loss is accompanied by the highest percentage of total nitrogen excreted in the form of allantoin-urea-ammonia, while the periods of greatest loss in body weight show the lowest per cent excreted in this form.

It is interesting to note the effect of the removal of yeast from the diet of both normal and deficient young animals. Removal of the yeast from the diet of the normal rats had no appreciable effect upon the per cent of nitrogen excreted as allantoin, urea, and ammonia (Table I). When yeast was removed from the diet of Animals 990 and 991 (Table II) which were suffering from acute xerophthalmia, both animals died at the end of 2 days. The absolute amount of allantoin-urea-ammonia remained very much as for the preceding period, but the per cent rose, due to lessened total nitrogen excretion, as only 1 gm. of basal diet had been ingested during the period. Since all the complications due to no food ingestion and death enter in this period, it is impossible to say what effect the removal of yeast would normally have had. For normal animals, however, it had practically no effect upon the per cent of total nitrogen excreted as allantoin, urea, and ammonia.

### *Allantoin Nitrogen.*

Only a few allantoin determinations have hitherto been made upon rat urine. Folin and Morris (8) studied the uric acid excretion of rats, but did not determine allantoin. Ackroyd (7) made six sets of determinations upon rats on a bread and water diet. The determinations were made over a 7 day period in each case, and the results given in amounts per rat, per day. These figures have here been recalculated in terms of number of gm. of allantoin excreted per kilo per day, and also in terms of the per cent of total nitrogen excreted as allantoin. Ackroyd and Hopkins (6) give figures for ten experiments upon young and adult rats fed bread and milk, or hydrolyzed casein diet with or without arginine, histidine, or tryptophane. Rose and Cook (11) have recently carried on a similar study. Typical results for animals on bread and milk or on whole casein diets are quoted below as most nearly

parallel with our determinations. When these results are compared with those found in the present study for similar animals on purified but adequate diet the amount of allantoin excreted per kilo of body weight will be seen to be of the same order. Since our young rats were in all cases smaller than those used by any of the other investigators our figures for total nitrogen are lower, but the allantoin coefficients (allantoin N per kilo per day) are considerably higher.

*Allantoin Excretion in the Rat.*

| Author. | No. of rats per cage. | Length of period. | Body weight. | Total N per rat per day. | Allantoin N per rat per day. | Allantoin N per kilo per day. | Total N excreted as allantoin. |
|---|---|---|---|---|---|---|---|
| | | *days* | *gm.* | *gm.* | *gm.* | *gm.* | *per cent* |
| Ackroyd (7). | 2 (adult). | 7 | 332 | 0.156 | 0.0087 | 0.052 | 5.6 |
| Ackroyd and Hopkins (6). | 2 (young, A, B). | 7 | 256 | 0.147 | 0.0116 | 0.089 | 7.8 |
| " " | 2 (young, M, N). | 7 | 204 | 0.170 | 0.0104 | 0.101 | 6.1 |
| " " | 2 (adult, AA, BB). | 7 | 411 | 0.200 | 0.0101 | 0.049 | 5.0 |
| Hunter, Givens, and Guion (9). | 11 (young). | 4 | 860 | 0.052 | 0.003 | 0.0382 | 5.7 |
| Rose and Cook (11). | 1 | 16 | 148 | 0.091 | 0.0057 | 0.038 | 6.5 |
| Present study (Table IV). | 2 (adult, F 8, F 26). | 2 | 225 | 0.094 | 0.011 | 0.050 | 11.7 |
| | | 2 | 205 | 0.091 | 0.018 | 0.087 | 19.7 |
| " " | 2 (young, 984, 989). | 3 | 226 | 0.032 | 0.0045 | 0.12 | 14.0 |
| " " | 2 (984, 988) | 3 | 144 | 0.041 | 0.0031 | 0.13 | 7.5 |
| " " | 2 (988, 989) | 3 | 198 | 0.069 | 0.006 | 0.18 | 8.7 |

In the case of adult and growing rats upon a diet adequate in vitamin A, and therefore representative of normal nitrogen

metabolism, without exception the per cent of total nitrogen recovered in the form of allantoin rises or falls with the per cent recovered as allantoin-urea-ammonia, and also with the gain or loss in body weight. In every case, the smaller the gain in weight, the higher the per cent of allantoin excreted and the larger the number of gm. per kilo of body weight. The averages for the various groups do not show this relationship in gm. of allantoin per kilo of body weight so steadily as do the individual periods for each group, due to variations in levels of excretion among the different subjects. The best comparisons, therefore, are between the different periods for the same group. When the animal is gaining in weight most rapidly, the per cent of total nitrogen excreted as allantoin is lowest. This is possibly explained upon the basis that, when growth takes place, ring compounds are necessary for new cell nuclear material, and so they are retained in the body. When growth is less, there is a correspondingly smaller amount of purines needed, and so more is excreted. When tissue destruction takes place, however, the nuclear material of the cells is set free and at least in part excreted, thus accounting for the higher excretion of allantoin during periods of loss in weight. When yeast was removed from the diet of young rats, the absolute amount of nitrogen excretion of all types fell, but remained qualitatively normal (Table I).

In the case of animals suffering from vitamin A deficiency the allantoin again keeps pace with the excretion of allantoin-urea-ammonia, but is inversely proportional to the gain in weight. The highest per cent excretion of allantoin takes place during periods of least loss in weight, and conversely, the lowest per cent of nitrogen is excreted in the form of allantoin when the animals are losing weight most rapidly (Table IV). The amount per kilo of body weight bears the same relation (Table IV). This is the reverse of conditions in normal metabolism.

The most plausible explanation seems to be that vitamin A is necessary for normal nitrogen metabolism in the rat, as suggested by Tschirch (5) for all vitamins, through its function as a ring closer in the formation of cyclic compounds in the body. In the case where temporary growth or maintenance occurred on the vitamin A-free diet, as for Rats 992, 993, 994, Period II (Table II), when a gain of 3 gm. was made, the percentage of total nitrogen

excreted as allantoin rises to the level seen in normal rats. The average for all growing rats upon a vitamin A-adequate normal diet was a gain of 4.5 gm. for the 3 day period and an allantoin percentage of 10.14 (Table IV), while the gain of Rats 992, 993, 994, Period II (Table II), on a vitamin A-free diet was 3 gm. and the allantoin excretion was 12.2 per cent, which appears to be a normal excretion. When, however, the animals lost weight as they did in all other periods, the percentage of allantoin becomes increasingly low. This would seem to show that the animal with no vitamin A in the diet cannot synthesize ring compounds, and that when tissue is broken down, as it is during periods of loss of weight, the ring compounds are not excreted but are retained by the body for reuse. This seems to be borne out by the additional fact that the whole tenure of allantoin excretion is lower than normal in animals deprived of vitamin A, and by the fact that the greater the loss the smaller the percentage of total nitrogen excreted as allantoin. As already mentioned, when yeast was removed from the diet of Rats 990 and 991, Period IV (Table II), both animals died after a period of 2 days. If we may conclude that the removal of the yeast had no more effect upon the per cent of allantoin excretion in this case than it had upon the normals (if there were an effect it would certainly decrease the percentage rather than increase it) the high percentage of allantoin, 15.10, excreted just before death may represent a suspended effort upon the part of the body to retain the ring compounds for reuse. For comparison, it is interesting to refer to Rats 984 and 988, Period II (Table I), where normal young rats lost 4 gm. of body weight, and excreted 12.02 per cent allantoin, which would seem to show that the percentage of 15.10 for a loss of 12 gm. was within the average range for normal rat metabolism. If so, then, as already suggested, perhaps just before death the animal reverts to its normal habit of discarding the ring compounds set free when body tissue breakdown occurs. As an illustration of allantoin excretion variations that are difficult to explain, Christman and Lewis (31) found that feeding amino acids to rabbits caused a decrease in the daily excretion of allantoin. They believe this is due to the fact that, in proportion to body weight, the amounts of amino acids administered were much larger than the amounts which caused an increased uric acid excretion in man, that they

are toxic in these large amounts, and consequently divert the course of normal purine metabolism.

The result here reported does not agree with those of Bickel and his coworkers, whose work was referred to above, for results obtained in avitaminosis (lack of all known vitamins) in dogs. They report a rise in allantoin excretion as the disease advances, with a return to normal just before death. Their work is reported, however, in absolute amounts, rather than in terms of the per cent of total nitrogen excreted, and this may account for the differences. They report marked fluctuations in allantoin excretion and this would be true of the work here reported if only absolute amounts were considered. When, however, these amounts are calculated in terms of the per cent of the total nitrogen excreted, they show relation to the amount of gain or loss in weight.

The fact that vitamins B and C as well as vitamin A were absent from the diet of these animals may well account for the differences in excretion reported. Experiments made in this laboratory upon rats fed vitamin B-deficient diets indicate an exaggeration of allantoin excretion which might counterbalance the allantoin economy enforced by vitamin A deficiency.

*Creatinine.*

In the adult animals, creatinine excretion runs parallel with the gain or loss in weight, and inversely proportional to the allantoin excretion. No definite relation to gain or loss in weight can be seen for the young animals.

Apparently, the young animals excrete a slightly smaller amount of creatinine nitrogen, 13.3 mg. per kilo per day, 2.87 per cent of total nitrogen, than do the adults, 14.2 mg. per kilo per day, 3.45 per cent total nitrogen, and the per cent is lower for rats suffering from the lack of vitamin A, 14.3 mg. per kilo per day, 2.37 per cent total nitrogen, than for normals, thus running parallel with allantoin excretion (Table IV).

The only comparable figures for creatinine excretion by rats are those furnished by Folin and Morris (8) and Rose and Cook (11). The former found for adult rats the average figure of 15.1 mg. per kilo per day and for young animals, 9.8. Rose and Cook found an average of 17 mg. per kilo per day for two growing rats fed a whole casein diet similar to that employed in our study.

### *Uric Acid.*

Uric acid excretion by the rat has been determined by Folin and Morris, Hunter, Givens, and Guion, and by Rose and Cook. Typical data from the work of each are quoted and recalculated in order to compare their results with those here reported.

*Uric Acid Excretion by the Rat.*

| Author. | No. of rats in cage. | Body weight. | Length of period. | Total N excreted. | Uric acid. | | |
|---|---|---|---|---|---|---|---|
| | | *gm.* | *days* | *gm.* | *mg.* | *mg. per kg. per day* | *per cent total N* |
| Hunter, Givens, and Guion (9) | 11 | 860 | 4 | 2.30 | 3.4 | 0.986 | 0.049 |
| Folin and Morris (8) | 1 | 290 | 1 | 0.173 | 2.07 | 7.20 | 0.40 |
| Rose and Cook (11) | 2 | 148 (average). | 16 | 1.47 | 13.2 | 5.56 | 0.3 |
| Present work (Rat F 8, Table III, average.) | 1 | 225 | 2 | 0.189 | 3.43 | 7.25 | 0.63 |

Our results for the excretion of uric acid per kilo of body weight, as well as the per cent of total nitrogen represented by uric acid nitrogen, are therefore fairly close to those reported by Folin and Morris, and by Rose and Cook, but the results of Hunter, Givens, and Guion seem much too low. It is to be remembered also that the other determinations quoted with the exception of those of Rose and Cook were made before the introduction of the more accurate modern methods for uric acid determination.

In normal adult rats, as found in the present study, the average uric acid excretion is 7.36 mg. per kilo of body weight per day, which represents 0.61 per cent of the total nitrogen excretion. It is lowest in amount with the greatest loss in weight, being parallel in this regard with creatinine, but inversely proportional to allantoin (Table III). When the percentage of allantoin is highest the uric acid excretion is lowest. Normal growing rats excrete an average of 7.44 mg. per kilo per day, or 0.58 per cent of total urinary nitrogen, which is almost identical with that of the adult. In normal growing rats there is no fixed relation

apparently, between allantoin and uric acid, but the excretion of uric acid in absolute amounts and in per cent remained remarkably constant, varying only within a range of 0.09 per cent. In young rats lacking vitamin A the excretion averages 10.09 mg. per kilo per day or 0.73 per cent, and in five out of six periods the uric acid excretion is again parallel with the loss in body weight as it is for normal adults, but it is directly proportional instead of inversely so, as it is for adults. Uric acid excretion for rats suffering from vitamin A deficiency is highest with the greatest loss in weight. Another curious feature is that allantoin and uric acid are still inversely instead of directly proportional. Apparently, then, the lack of vitamin A does not decrease the excretion of uric acid, but rather increases it as the proportion of total nitrogen derived from breakdown of body tissues (and therefore purine compounds) rises. This suggests that the animals upon a vitamin A-free diet cannot reuse the unoxidizable portions of the purine compounds set free by tissue breakdown, but excrete them as uric acid in proportion as the loss in weight sets these ring compounds free. Apparently, then, the lack of vitamin A prevents the normal synthesis of ring compounds in the body, and when the disease has advanced to the stage where loss of weight takes place the portions of the nuclear material which are normally oxidizable to allantoin are partially retained for reuse in the body, while those portions which normally are unoxidizable and which are excreted as uric acid are not of such nature that they can be used over again in the body, and so they are discarded through the urine. The work of Christman and Lewis (31) already mentioned, shows that the two purine catabolites, allantoin and uric acid, do not necessarily behave alike. Starkenstein (32) working with the effects of atophan caused a decrease in the elimination of allantoin in the rabbit and an increase in uric acid in man. Apparently, judging from these instances, disturbances in diet frequently cause a lowering of allantoin excretion and increase in uric acid, as is here reported for vitamin A deficiency.

In the experiments of Christman and Lewis on rabbits uric acid as well as allantoin determinations were made in one series only. It is interesting to note that in the case of three animals so studied the uric acid is usually considerably increased when the allantoin is decreased following the administration of gelatin, urea, or

glycocoll. This agrees with our theory of the reuse of allantoin fragments under certain conditions and the continued discarding of uric acid, if it can be assumed that the amino-containing substances fed by Christman and Lewis were utilized in tissue anabolism along with molecules ordinarily oxidized to allantoin.

SUMMARY.

1. The partition of urinary nitrogen as to urea and ammonia, allantoin, uric acid, and creatinine has been determined for nine periods of 3 days each upon three young rats fed a diet adequate in all respects. The per cent of recovery of total nitrogen in the forms mentioned varied between 45 and 64. The relative amounts of allantoin *decreased* with *gain* in body weight and *increased* with *loss* in body weight. The average was 10.14 per cent of nitrogen as allantoin with average gain of 4.5 gm. of body weight per period, or 0.144 gm. of allantoin N per kilo of body weight, correlated with a gain of 28.8 gm. in body weight. The average amount of nitrogen excreted as uric acid was 0.58 per cent and of creatinine 2.87.

2. The partitition of urinary nitrogen has been determined in the same way for nine periods of 3 days each upon nine young rats fed a diet adequate in all respects except as to vitamin A. All of these animals showed the usual symptoms of vitamin A deficiency, such as declining weight and ophthalmia. The per cent of recovery of total nitrogen in the forms mentioned ranged between 46 and 59. The relative amounts of allantoin *increased* with *gain*, or in most cases lessened *loss*, in body weight, and *decreased* with increased *loss* in body weight. The average was 8.31 per cent of nitrogen as allantoin with average loss of 5.1 gm. of body weight or 0.165 gm. of allantoin N per kilo of body weight, correlated with an average *loss* of 32.8 gm. of body weight. The average amount of nitrogen excreted as uric acid was 0.73 per cent, and as creatinine 2.37.

3. The partition of urinary nitrogen has been similarly determined for six periods of 2 days each on two adult female rats fed an adequate diet. The range of recovery was 51 to 81 per cent of total urinary nitrogen, which are definitely larger figures than those found in either group of young animals. The relative excretion of allantoin varied inversely with gain in weight as was

the case with the young rats on adequate diet. The average was 14.02 per cent of nitrogen excreted as allantoin with average *gain* of body weight of 2.1 gm., or 0.137 gm. of allantoin N per kilo of body weight, correlated with a *gain* of 15.8 gm. in body weight. This may be contrasted with the figure 8.31 per cent and 0.165 gm. per kilo with 5.1 gm. *loss* in weight found for vitamin A-deficient young rats. The average amount of nitrogen excreted as uric acid was 0.61, as creatinine 3.45.

4. A theory is advanced to account for these differences between the excretion of allantoin and uric acid in normal and vitamin A-deficient animals. It is suggested that in the absence of vitamin A the animal organism fails to produce purine-containing compounds from the ordinary sources, perhaps arginine and histidine, but continues to utilize over again such portions of discarded purine ring-containing substances as are ordinarily excreted in the form of allantoin. The portions of these compounds which are oxidized only to uric acid are apparently no longer usable and continue to be excreted in proportion to the amount of destruction of cellular material in vitamin A-deficient as in normal animals.

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# THE RELATION BETWEEN THE AMOUNT OF ULTRA-VIOLET LIGHT RECEIVED BY HENS AND THE AMOUNT OF ANTIRACHITIC VITAMIN IN THE EGGS PRODUCED.*

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In the conclusion of an article on leg weakness in growing chicks Hughes, Payne, and Latshaw[1] state: "The variation in the hatchability of the eggs produced by hens receiving varying amounts of ultra-violet light is perhaps due to a variation in the antirachitic vitamin content of the egg. This is merely an assumption but points the way to some very interesting research work." This year experiments have been completed in which the relative antirachitic vitamin content of eggs from hens receiving different amounts of ultra-violet light has been determined, and, as predicted last year, the hens which receive an abundance of ultra-violet light are able to put an abundance of the antirachitic vitamin in their eggs, while the hens which receive a very limited amount of ultra-violet light are able to put only a small amount of the antirachitic vitamin in their eggs.

In this experiment eggs from four pens of hens receiving varying amounts of ultra-violet light were tested by feeding the eggs to growing chicks. These four pens of hens were housed in a shed-roof type house with a south exposure. Each pen was 8 by 10 feet and had a glass window in the south side 3 by 8 feet. Pens I and III were allowed to run in a small yard south of the house

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[1] Hughes, J. S., Payne, L. F., and Latshaw, W. L., The influence of ultra-violet light on leg weakness in growing chicks and on egg production, *Poultry Sc.*, 1925, iv, 151.

where they had access to direct sunlight. The windows in Pens II and IV were kept closed at all times, so all the sunlight received by these pens was filtered through glass. Pens I and II received a 30 minute treatment each day of ultra-violet light from a quartz mercury arc lamp. In this way Pen I received both the direct sunshine and ultra-violet light from the mercury arc lamp. Pen II received sunlight filtered through glass and light from the mercury arc lamp. Pen III received direct sunshine, while Pen IV received only the glass-filtered sunshine. All these pens received the regular Kansas State Agricultural College ration which includes a liberal supply of green sprouted oats through the winter months and green alfalfa during the summer months. The hens were placed in the experiment October 1, 1924, and the study to determine the relative vitamin content of the eggs was begun 6 months later, April 1, 1925.

Four lots of growing chicks were used to test the relative antirachitic vitamin content of the eggs from these four pens of hens. Ten 1 week old White Leghorn chicks were used for each lot. They were placed in small pens (24 by 30 inches) in the nutrition room in such a way that all the lots received the same amount of light but none of them received any direct sunlight through the window. Each lot received the same basal ration which consists of yellow corn 80 parts, meat scrap 10 parts, and middlings 10 parts. This particular basal ration was chosen as a number of previous experiments had shown that when 1 week old chicks were placed on it under the lighting condition in our laboratory, they would develop unmistakable signs of rickets in from 4 to 6 weeks, while if a supplementary feed containing a liberal amount of the antirachitic vitamin was added to it, the chicks would develop normally.

In addition to this basal ration each lot received one egg a day from one of the pens of hens. Lot I of chicks received one egg a day from Pen I of hens, Lot II of chicks received one egg a day from Pen II of hens, etc. The shells of the eggs were not used. The raw white and yolk of an egg were mixed fresh each morning with the amount of basal rations that the lot would utilize during the day. Each lot was allowed to consume as much of the basal ration as they would each day after they had eaten the portion which had been mixed with the egg.

TABLE I.

*Final Weights of Chicks Receiving Eggs from Hens Which Had Received Varying Amounts of Ultra-Violet Light.*

| Lot No. | Kind of egg received. | Average final weight. |
|---|---|---|
| | | gm. |
| I | Egg from hens receiving direct sunlight + 30 min. ultra-violet irradiation per day. | 450 |
| II | Egg from hens receiving 30 min. ultra-violet irradiation and sunlight through glass window. | 390 |
| III | Egg from hens receiving direct sunshine. | 473 |
| IV | Eggs from hens receiving only sunshine filtered through glass windows. | 294 |

TABLE II.

*Chemical Analysis of Bones of Chicks Receiving Eggs from Hens Which Had Received Varying Amounts of Ultra-Violet Light.*

| Lot No. | Calcium. | Phosphorus. | Ether extract. | Ash. |
|---|---|---|---|---|
| | *per cent* | *per cent* | *per cent* | *per cent* |
| I | 20.54 | 9.20 | 16.89 | 51.4 |
| II | 19.25 | 8.70 | 5.95 | 48.1 |
| III | 20.05 | 9.06 | 16.50 | 49.5 |
| IV | 15.73 | 7.32 | 1.51 | 40.7 |

The per cents of calcium, phosphorus, and ash are based on the ether-extracted material.

TABLE III.

*Calcium and Inorganic Phosphorus of Blood from Chicks Receiving Eggs from Hens Which Had Received Varying Amounts of Ultra-Violet Light.*

| Lot No. | Plasma calcium. | Inorganic phosphorus. | Condition of chick. |
|---|---|---|---|
| | *mg. per 100 cc.* | *mg. per 100 cc.* | |
| I | 12.9 | 5.00 | Normal. |
| II | 11.5 | 3.40 | Slight rickets. |
| III | 13.0 | 5.00 | Normal. |
| IV | 9.67 | 3.72 | Rickets. |

Each chick was wing banded. They were weighed individually each week (Table I shows the final average weight) and notes on the general condition of each chick were kept after signs of rickets developed.

The experiment was discontinued at the end of 10 weeks, the chicks then being 11 weeks old. At this time pictures were taken showing the general condition of each lot, after which the chicks were killed for bone and blood analysis. Three bones (femur, tibia, and humerus) from each chick in a lot were carefully cleaned and composited into one sample which was analyzed for ether extract, ash, calcium, and phosphorus (Table II). Approximately 4 cc. of blood were drawn from the heart of each chick in a lot and composited into one sample which was analyzed for calcium and inorganic phosphorus (Table III).[2]

The growth of the chicks, the general condition of the lots at the end of the experiment, the bone analysis, and the blood analysis show that the eggs from hens receiving an abundance of ultra-violet light contain an abundance of the antirachitic vitamin, while eggs from hens receiving a limited amount of ultra-violet light contain a relatively smaller amount of the antirachitic vitamin. Whether or not the 30 minute treatment of ultra-violet light from the mercury arc lamp which Pen I of hens received enabled them to store more of the antirachitic vitamin in their eggs than Pen III which received only the direct sunlight is not shown by this experiment as the one egg a day from Pen III seemed to contain a sufficient amount of the antirachitic vitamin for the ten chicks.

The 30 minute ultra-violet treatment which Pen II of hens received did not enable them to store sufficient antirachitic vitamin in their eggs to entirely prevent the development of rickets in Lot II of the chicks. There was sufficient antirachitic vitamin in their eggs, however, to reduce very materially the severity of rickets produced.

All the chicks in Lot IV developed severe rickets. This shows that the eggs from Pen IV of hens which were receiving only sun-

[2] The blood calcium was determined by the modified Tisdall method. Clark, E. P., and Collip, J. B., A study of the Tisdall method for the determination of blood serum calcium with a suggested modification, *J. Biol. Chem.*, 1925, lxiii, 461.

The blood phosphorus was determined by the Briggs modification of the Bell-Doisy method. Briggs, A. P., A modification of the Bell-Doisy phosphate method, *J. Biol. Chem.*, 1922, liii, 13. Briggs, A. P., Some applications of the colorimetric phosphate method, *J. Biol. Chem.*, 1924, lix, 255.

light which had been filtered through glass contained very little of the antirachitic vitamin. Four of the chicks in this lot developed tetany. When excited they would go into spasms which would last from 2 to 5 minutes. It will be noted (Table III) that the calcium content of the blood of the chicks in this lot was lower than that for normal chicks. It has been found by a number of workers[3] that when infants suffering from rickets develop tetany their blood calcium is usually much lower than normal.

The hatching record of the eggs (Table IV) from the three lots

TABLE IV.

*Hatching Record of Eggs from Hens Receiving Varying Amounts of Ultra-Violet Light.*

| Lot No. | Light received. | No. of fertile eggs set Jan. 1–June 30. | Per cent of fertile eggs hatched. |
|---|---|---|---|
| I | Direct sunshine plus 30 min. ultra-violet light per day. | 554 | 67.43 |
| II | 30 min. ultra-violet light per day plus sunshine through glass window. | 846 | 71.63 |
| III | Direct sunshine. | 672 | 75.45 |
| IV | Sunshine through glass window. | 673 | 52.9 |

of hens is interesting, as it shows that the eggs with the low antirachitic vitamin content hatch only 53 per cent, while those with a higher antirachitic vitamin content hatch an average of over 70 per cent. We have found that day old chicks hatched from the eggs having a low antirachitic vitamin content contained less calcium in their bodies than day old chicks hatched from eggs having a high antirachitic vitamin content. This indicates that disturbances in the mineral metabolism may begin before the chick is hatched.

[3] Kramer, B., Tisdall, F. F., and Howland, J., Observations on infantile tetany, *Am. J. Dis. Child.*, 1921, xxii, 431. Howland, J., and Kramer, B., Calcium and phosphorus in the serum in relation to rickets, *Am. J. Dis. Child.*, 1921, xxii, 105. Dunham, E. C., Rickets in an infant of thirty-four days, *Am. J. Dis. Child.*, 1923, xxvi, 155.

## CONCLUSIONS.

The amount of ultra-violet irradiation which a hen receives is an important factor in determining the antirachitic vitamin content of the eggs which she produces when her feed is low in the antirachitic vitamin.

Eggs which have a low antirachitic vitamin content do not have as high a hatchability as eggs having a high antirachitic vitamin content, other factors influencing hatchability being constant.

# THE DETERMINATION OF CALCIUM IN TISSUES, FECES, AND MILK.

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The determination of the inorganic constituents of tissues is almost invariably carried out on the ash.

Ashing, even in the hands of the skilful and experienced chemist, is at best a laborious and time-consuming procedure, and, in addition, may be considered as a distinctly expensive method on account of the platinum or silica ware which must be used.

For some time we have been using in this laboratory autoclave digestion of tissues as a substitute for ashing, and believe that we have found in this procedure a technique which permits of accurate determination of several of the inorganic constituents, and which is economical both of apparatus and of the time of the analyst.

In this paper we wish to report the results obtained by the application of autoclave digestion in the determination of calcium in tissues, feces, and milk. The use of the autoclave in the determination of other inorganic constituents of tissues will be taken up in subsequent publications.

The procedure is as follows:

*Method for the Determination of Calcium in Tissues.*

10 gm. of finely ground tissue together with 50 cc. of 0.1 N sodium hydroxide are placed in a large Pyrex test-tube (25 × 200 mm.) graduated at 60 cc. The tube is covered with tin-foil and autoclaved for 2 hours at a temperature of 180°C. by which treatment practically complete solution is effected. The solution is now rendered strongly acid by the addition of concen-

trated hydrochloric acid, diluted with distilled water to a volume of 60 cc., allowed to stand for at least 30 minutes, and then filtered by suction through a Gooch crucible provided with a mat of calcium-free asbestos fiber or filter paper. We sometimes centrifuge the liquid before attempting to filter, a procedure which while not essential greatly facilitates the latter operation.

At this stage the solution should be clear and absolutely free from suspended particles; the color, however, varies greatly from the light yellow given by muscle to the dark reddish brown noted when liver is used. An aliquot of the solution (usually 15 cc. in the case of normal tissues) is measured into a 50 cc. conical centrifuge tube, a half volume of 4 per cent ammonium oxalate solution is added, and the reaction is made alkaline to methyl red by the cautious addition of concentrated ammonium hydroxide. This precipitation should be made shortly after filtration, as if the filtrate is allowed to stand too long the color gradually deepens and the further procedure becomes more difficult. After standing for at least 1 hour the precipitate is centrifuged down, and the supernatent liquid is poured off as completely as possible without disturbing the precipitate.

This precipitate is now dissolved in 5 cc. of approximately normal sulfuric acid and titrated at room temperature with 0.1 N potassium permanganate until pink, and after a period of 5 to 10 minutes there are added 5 cc. of a 4 per cent solution of ammonium oxalate. As soon as the solution is perfectly clear 5 cc. of water are added, and the reaction is adjusted to the neutral point of methyl red by the cautious addition of concentrated ammonium hydroxide, using approximately normal hydrochloric acid for back titrations if the reaction has passed the neutral point.

After standing for 1 hour or longer if convenient the precipitate is centrifuged down, the supernatant liquid poured off as completely as possible, and the calcium oxalate washed with 30 to 35 cc. of ice-cold water, centrifuged again, and after decantation of the wash water, the precipitate is dissolved in 5 cc. of approximately normal sulfuric acid, the tube placed for 5 minutes in a water bath at 70°C., and the solution then titrated with 0.01 N potassium permanganate.

The blank titration given by the sulfuric acid is subtracted.

Since 1 cc. of 0.01 N potassium permanganate is equivalent to 0.2 mg. of calcium, the calculation is as follows:

$$\text{mg. per 100 gm. sample} = \text{titration} \times \frac{0.2 \times 60}{\text{aliquot}} \times \frac{100}{\text{sample in gm.}}.$$

Using a 10 gm. sample of tissue and a 15 cc. aliquot this equals the titration times 8.

A few words regarding our reasons for certain of the procedures described above may not be out of place at this point.

During the early stages of the work we felt that in view of the possibility and desirability of using the same general procedure for the determination of all of the inorganic constituents, hydrochloric acid was the best reagent with which to autoclave the tissues. A great number of different concentrations and different temperatures were employed, but with generally unsatisfactory results. For some reason, possibly the formation of humin substances, at later stages of the procedure there tended to appear organic precipitates generally dark in color, and although the intermediate oxidation with potassium permanganate will remove traces of organic matter, it is felt that the appearance of such large quantities is too great an obstacle to surmount even with this treatment.

Sodium hydroxide was found to be more satisfactory; there must, however, be a rough parallelism between the amount of protein present and the amount of this reagent used, otherwise the large excess of alkali at the high temperature used attacks the glass with the formation of silicates that precipitate as silicic acids in acid solution.

It would undoubtedly be desirable to have a procedure in which a single precipitation is used, and frequently it has been found that the second precipitation is unnecessary because of the cleanness of the first precipitate. Since, however, such satisfactory conditions were not obtained consistently, it has been deemed advisable to introduce the double precipitation with the intermediate oxidation by means of potassium permanganate. Under these conditions the permanganate destroys any traces of organic matter that may have been carried down with the oxalate.

Shohl (1) states that he attempted to employ a permanganate oxidation to destroy the organic material of the urine, but found

such a procedure impracticable because the large quantities of manganous ions formed were difficult to hold in solution. We have not encountered a similar difficulty due to the relatively small amounts of the reagent used. However, when even somewhat larger amounts were treated with ammonium oxalate and the reaction adjusted to the neutral point of methyl red, not the slightest turbidity developed, nor was there a detectable precipitate when centrifuged.

Shohl has shown that the reaction for the quantitative precipitation of calcium as the oxalate, in the presence of possible interfering substances, such as magnesium and phosphates, as determined and applied empirically by McCrudden (2), is obtained at the neutral point of the indicator methyl red at a pH between 4.8 and 5.4, and we have therefore attempted to make all precipitations at this reaction.

Comparison of results obtained by the ashing and the autoclaving methods are given in Table I. Table II presents the figures for the recovery of added calcium. The results obtained are, we feel, within the limits of reasonable accuracy, when the fact is taken into consideration that even finely ground tissues are not homogeneous, so that it is practically impossible to obtain two samples of like composition.

The ashing used as a control method was carried out in silica dishes which were ignited in a muffle-furnace heated by gas.

The ash was extracted with normal hydrochloric acid and the calcium precipitated by a half volume of 4 per cent ammonium oxalate, the reaction being adjusted to the neutral point of methyl red. After about 1 hour's standing, the precipitate was centrifuged, washed, and titrated with 0.01 N $KMnO_4$ in the usual manner.

For the recovery experiments standard solutions of $CaCl_2$ which were standardized by the oxalate method were used.

In order to insure absolute freedom from calcium the ammonium hydroxide and hydrochloric acid were redistilled. The ammonium oxalate was recrystallized twice from water, while the sodium hydroxide was made from metallic sodium. Considering the quantities used and the greatest possible contamination in high grade products, we feel that the amount of calcium that could possibly be introduced by potassium permanganate

TABLE I.

*Results Obtained on Tissues by Ashing and by the Autoclave Method.*

| Tissue. | Ca per 100 gm. tissue. | |
|---|---|---|
| | Ashing method. | Autoclave method. |
| | *mg.* | *mg.* |
| Beef muscle. | 10.72 | 11.04 |
| | 10.72 | |
| | 10.92 | 10.80 |
| | 11.12 | 11.04 |
| Beef liver. | 8.04 | 8.12 |
| | 7.88 | 8.04 |
| | 7.96 | 8.20 |
| | 7.92 | 8.16 |
| Muscle, dog. | 5.90 | 5.98 |
| Liver, " | 26.40 | 27.70 |
| Lungs, " | 40.80 | 46.20 |
| Kidneys, " | 40.0 | 41.3 |
| Lungs, " | 105.9 | 100.3 |

TABLE II.

*Results Obtained in the Recovery of Calcium Added to Tissues (Autoclave Method).*

| Tissue. | Ca in amount of tissue used. | Ca added. | Ca found. | Ca recovered. | |
|---|---|---|---|---|---|
| | *mg.* | *mg.* | *mg.* | *mg.* | *per cent* |
| Beef muscle. | 0.274 | 0.255 | 0.516 | 0.242 | 95.0 |
| | 0.274 | 0.255 | 0.531 | 0.257 | 100.8 |
| | 0.274 | 0.255 | 0.524 | 0.250 | 98.0 |
| | 0.274 | 0.510 | 0.760 | 0.486 | 95.3 |
| | 0.274 | 0.510 | 0.770 | 0.496 | 97.4 |
| Beef liver. | 0.204 | 0.126 | 0.325 | 0.121 | 96.0 |
| | 0.204 | 0.126 | 0.326 | 0.122 | 96.8 |
| | 0.204 | 0.126 | 0.331 | 0.127 | 100.8 |
| | 0.204 | 0.126 | 0.328 | 0.124 | 98.5 |
| Muscle, dog. | 0.460 | 0.320 | 0.780 | 0.320 | 100.0 |
| Liver, " | 0.438 | 0.320 | 0.768 | 0.330 | 103.1 |
| | 1.656 | 0.320 | 1.974 | 0.318 | 99.4 |
| Kidney, " | 0.530 | 0.320 | 0.846 | 0.316 | 98.7 |
| | 0.372 | 0.320 | 0.692 | 0.320 | 100.0 |
| Lungs, " | 0.576 | 0.320 | 0.688 | 0.316 | 97.5 |
| Brains, " | 0.234 | 0.320 | 0.552 | 0.318 | 99.4 |
| | 0.230 | 0.320 | 0.549 | 0.319 | 99.6 |
| | 0.234 | 0.320 | 0.549 | 0.315 | 98.5 |

and sulfuric acid is negligible. As a final precaution blanks were run on each of the reagents.

The asbestos used for filtration was a high grade, long fiber

TABLE III.

*Results on the Determination of Calcium in Milk by the Autoclave and by the Ashing Methods.*

| Milk. | Ca per 100 cc. milk. | |
|---|---|---|
| | Ashing method. | Autoclave method. |
| | *mg.* | *mg.* |
| Cow. | 122.0 | 122.8 |
| | 119.0 | 123.2 |
| | 122.0 | 123.2 |
| | 122.0 | 123.6 |
| Human. | 26.7 | 26.8 |
| | 26.4 | 26.7 |
| | 26.8 | 26.1 |
| | 26.9 | 26.9 |

TABLE IV.

*Recovery of Calcium Added to Cow's Milk (Autoclave Method).*

| Ca in volume of milk used. | Ca added. | Ca found. | Ca recovered. | |
|---|---|---|---|---|
| *mg.* | *mg.* | *mg.* | *mg.* | *per cent* |
| 0.312 | 0.160 | 0.468 | 0.156 | 97.5 |
| 0.312 | 0.160 | 0.472 | 0.160 | 100.0 |
| 0.312 | 0.160 | 0.474 | 0.162 | 101.3 |
| 0.312 | 0.160 | 0.472 | 0.160 | 100.0 |
| 0.418 | 0.337 | 0.755 | 0.337 | 100.0 |
| 0.418 | 0.337 | 0.770 | 0.352 | 104.3 |
| 0.418 | 0.337 | 0.765 | 0.347 | 103.0 |
| 0.335 | 0.252 | 0.589 | 0.254 | 100.8 |
| 0.335 | 0.252 | 0.588 | 0.253 | 100.4 |
| 0.335 | 0.252 | 0.594 | 0.249 | 98.8 |
| 0.335 | 0.252 | 0.593 | 0.248 | 98.4 |
| 0.335 | 0.126 | 0.462 | 0.127 | 100.8 |
| 0.335 | 0.126 | 0.466 | 0.131 | 104.0 |

"acid-extracted" product which was further treated by boiling for some hours in strong hydrochloric acid and was subsequently washed in distilled water until free from chlorides.

*Method for the Determination of Calcium in Milk and in Feces.*

The autoclave method has been applied successfully to the determination of calcium in milk and in feces, although on account of the smaller amount of protein and other hydrolyzable organic matter present it was found necessary to modify the procedure somewhat.

1 cc. of cow's milk, 5 cc. of human milk, or 1 gm. of feces, is introduced into a large test-tube and autoclaved for 1 hour at 160°C. with 50 cc. of 0.01 N sodium hydroxide solution. The

TABLE V.

*Results Obtained on the Determination of Calcium in Feces by Ashing and by the Autoclave Method.*

| Ca per 100 gm. feces. | |
|---|---|
| Ashing method. | Autoclave method. |
| *mg.* | *mg.* |
| 141.0 | 139.8 |
| 139.5 | 139.2 |
| 138.2 | 145.2 |
| 138.4 | 145.2 |

TABLE VI.

*Recovery of Calcium Added to Feces (Autoclave Method).*

| Ca in the portion of feces used. | Ca added. | Ca found. | Recovery. | |
|---|---|---|---|---|
| *mg.* | *mg.* | *mg.* | *mg.* | *per cent* |
| 0.243 | 0.336 | 0.581 | 0.338 | 100.5 |
| 0.348 | 0.336 | 0.681 | 0.333 | 99.0 |
| 0.253 | 0.336 | 0.591 | 0.338 | 100.5 |
| 0.243 | 0.336 | 0.583 | 0.340 | 99.4 |

remainder of the procedure is identical with that described for muscle.

In Tables III to VI inclusive we have collected some of the analytical results obtained by the use of this technique.

*Method for the Determination of Calcium in Bone.*

We have obtained excellent results by the use of the autoclave in the determination of calcium in bone. The procedure de-

scribed is adapted to the analysis of one entire bone of a small animal, as only in this way it is possible to obtain a representative sample of this tissue. After the broken bone (in weight from 5 to 10 gm.) has been digested in the autoclave with 50 cc. of tenth normal sodium hydroxide, the alkaline liquid is carefully poured off, and the remaining inorganic framework is dissolved by the addition of about 15 cc. of concentrated hydrochloric acid. The acid and alkaline extracts are combined, diluted to a known volume, and the calcium determined in an aliquot by the procedure described for the other tissues.

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# A STUDY OF THE EFFECT OF EXCESSIVE CALCIUM INGESTION ON THE CALCIUM CONTENT OF TISSUES WITH AND WITHOUT THE APPLICATION OF ULTRA-VIOLET LIGHT.

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Within the past 10 years numerous investigations have been made with a view of determining the effect of the administration of calcium salts on the concentration of this element in the blood (1), and since the part played by ultra-violet light in connection with bone growth has been recognized, the effect on blood calcium of exposure to the mercury or the carbon arc with and without high calcium intake has also received consideration (2).

As a result of the earlier work the view has, we believe, been held almost unanimously by investigators working in the field of inorganic metabolism that the administration by mouth of considerable quantities of calcium salts was without effect on the level of blood calcium, except in cases where the calcium level was below the normal, as in acute rickets (3), in which event the oral administration of some salts of this element has been shown to cause an increase in the serum calcium to the normal concentration. More recent investigators are, however, not in entire agreement with these conclusions, as results have been published (4) indicating that while calcium lactate, administered by mouth, is without effect on the blood calcium level, the administration of calcium chloride to men, to infants, and to dogs may frequently, but not invariably, cause an increase of as much as 20 per cent in the level of the serum calcium.

While our views regarding the effect of a high level of calcium intake have been considerably clarified as regards the blood, by

the work cited above, we still know but little regarding the results produced by an increased calcium intake on the tissues other than bone.

Heubner and Rona (5) have carried out an extensive series of analyses to determine the calcium content of the tissues of nine normal cats, of six cats suffering from "acute" calcium poisoning, and of four animals in which "chronic" calcium poisoning had been induced. The calcium was administered subcutaneously in the form of calcium chloride; for the production of "acute" intoxication the animals received 0.3 to 1.2 gm. $CaCl_2 \cdot 6H_2O$ per kilo given in several injections during the 24 hours; for the production of the "chronic" condition a daily dose of the same salt amounting to 0.7 gm. per kilo was administered for periods varying from 9 to 49 days.

Analysis of the tissues of these animals indicated that the calcium concentration remained well within the limits found by these investigators for normal dogs. It was noted, however, that the variations in the calcium content of normal tissues are very great, sometimes amounting to several hundred per cent, a finding which we have confirmed in the work reported in this paper. The explanation of these large variations lies, we believe, in the difficulty, one may almost say the impossibility, of obtaining samples of "pure" tissue; for example, muscle taken from different animals under identical conditions may, and probably always does, contain varying amounts of blood, lymph, connective tissue, fat, and water. This difficulty has been recognized by Shepard (6) who has attempted to eliminate this source of error by the combination of chemical and histological methods.

In the experimental work described below we have attempted to observe the effect, if any, produced on the tissues of animals which were fed daily for several weeks excessive doses of calcium salts. Rabbits were used as experimental subjects. The animals were purchased from several local dealers, and after receipt at the laboratory were kept for 5 weeks before being used for experimental purposes, during which period they were fed a ration of oats, hay, and water. Each rabbit occupied a separate cage, in size approximately about 2 feet square. The calcium salt administered was given intimately mixed with about 75 gm. of finely ground carrot. This mixture, which was readily taken

TABLE I.

*Calcium in Tissues and Blood after the Administration of Calcium.*

| Rabbit No. | Sex. | Weight. | Calcium per 100 gm. | | | | | | Per 100 cc. serum. | | Remarks. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Muscle. | Liver. | Lung. | Kidney. | Brain. | Bone. | Ca | Inorganic P. | |
| | | *gm.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *gm.* | *mg.* | *mg.* | |
| 1 | ♀ | 2700 | 9.32 | 5.16 | | | | 12.63 | | 3.9 | Rabbits 1 to 7 inclusive received 1.0 gm. calcium lactate per day. |
| 2 | ♀ | 1700 | 8.68 | | | | | | | 4.0 | |
| 3 | ♀ | 2300 | 5.91 | 5.31 | | | | 12.5 | | 3.9 | |
| 4 | ♀ | 3000 | 7.36 | 7.43 | 10.75 | 8.05 | 15.35 | 13.8 | | | Figures for lung, kidney, and brain are for a composite of the organs of Rabbits 1, 3, and 4. Figures for lung, kidney, and heart are for a composite of the organs of Rabbits 5 and 7. |
| 5 | ♂ | 2100 | 4.58 | | | | | | | | |
| 6 | ♀ | 2100 | 5.78 | 12.95 | | | | | | | |
| 7 | ♂ | 2600 | 5.54 | | 11.51 | 11.88 | | | | | |
| 8 | ♀ | 3400 | 6.73 | 9.15 | | | | 12.7 | 15.3 | 3.7 | Rabbits 8 to 12 inclusive received 0.5 gm. $CaCl_2$ per day. |
| 9 | ♀ | 2500 | 6.00 | 9.15 | | | | 13.2 | 14.0 | 3.7 | |
| 10 | ♂ | 2500 | | 8.80 | | | | 11.8 | 12.6 | 4.1 | |
| 11 | ♂ | 3200 | 6.62 | | | | | 13.1 | 15.3 | 4.0 | |
| 12 | ♀ | 3200 | 9.25 | 7.71 | | | | 11.6 | 13.2 | 4.0 | |
| 13 | ♀ | 2300 | 5.30 | 6.23 | | | | 16.15 | 15.0 | 5.4 | Rabbits 13 to 18 inclusive received no calcium salts. |
| 14 | ♀ | 2100 | 6.05 | 6.09 | | | | 14.78 | 15.0 | 5.1 | |
| 15 | ♀ | 2300 | 3.99 | 5.83 | | | | 15.17 | 14.1 | 5.0 | |
| 16 | ♂ | 1400 | 5.43 | 7.83 | | | | | 15.6 | 5.0 | |
| 17 | ♀ | 2700 | 8.37 | 11.60 | | | | 15.70 | 14.9 | 4.4 | |
| 18 | ♀ | 2800 | 7.48 | 7.46 | | | | 12.31 | 15.3 | 4.6 | |

TABLE II.

*Calcium in Tissues and Blood Serum after the Administration of Calcium Salts and Exposure to Ultra-Violet Light.*

| Rabbit No. | Sex. | Weight. | Calcium per 100 gm. | | | | | | | Per 100 cc. serum. | | Remarks. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Muscle. | Liver. | Lung. | Kidney. | Brain. | Heart. | Bone. | Ca | Inorganic P. | |
| | | *gm.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *gm.* | *mg.* | *mg.* | |
| 13 | ♂ | 1600 | 5.13 | 8.25 | | | | | 19.80 | 15.1 | 5.64 | Rabbits 13–30 inclusive received 1.0 gm. calcium lactate per day. |
| 14 | ♀ | 1700 | 7.42 | 7.42 | 16.60 | 11.50 | | 12.78 | 24.35 | 15.1 | 5.08 | Figures on lung, kidney, brain, and heart are on combined organs of Rabbits 13 and 14. |
| 15 | ♂ | 2300 | 6.38 | 6.45 | 11.9 | 8.30 | 11.8 | 6.18 | 23.7 | 15.2 | 5.08 | Figures on lung, kidney, brain, and heart are on combined organs of Rabbits 15 and 17. |
| 17 | ♂ | 1900 | 5.58 | 5.77 | | | | | 20.0 | 16.0 | 5.64 | |
| 18 | ♂ | 1800 | 8.98 | 7.23 | 7.08 | 8.10 | 8.60 | 10.18 | 20.9 | 16.0 | 5.17 | Figures on lung, kidney, brain, and heart are on combined organs of Rabbits 18 and 29. |
| 28 | ♂ | 1800 | 6.37 | 6.61 | 9.21 | 10.65 | 8.21 | 11.72 | 22.2 | 15.3 | 5.17 | Figures on lung, kidney, brain, and heart are on combined organs of Rabbits 28 and 30. |
| 29 | ♂ | 2000 | 8.58 | 6.52 | | | | | 21.2 | 15.0 | 5.08 | |
| 30 | ♀ | 1500 | 7.13 | 6.43 | | | | | 22.2 | 15.3 | 5.16 | |
| 19 | ♂ | 1700 | 5.97 | 7.80 | 6.62 | 6.84 | 7.27 | 7.85 | 19.6 | 13.5 | 4.2 | Rabbits 19–27 inclusive received 0.5 gm. calcium chloride per day. |
| 20 | ♂ | 1900 | 5.94 | 5.68 | | | | | 17.51 | 13.5 | 4.2 | Figures on lung, kidney, brain, and heart are on combined organs of Rabbits 19 and 21. |
| 21 | ♂ | 1800 | 6.60 | 8.74 | | | | | 16.20 | 14.0 | 4.2 | |
| 22 | ♂ | 2400 | 6.42 | 6.38 | 10.82 | 8.79 | 5.63 | 8.12 | 18.22 | 16.0 | 3.3 | Figures on lung, kidney, brain, and heart are on combined organs of Rabbits 20 and 22. |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 22 | ♂ | 2400 | 6.42 | 6.38 | 10.82 | 8.79 | 5.63 | 8.12 | 18.22 | 16.0 | 3.3 | Figures on lung, kidney, brain, and heart are on combined organs of Rabbits 20 and 22. |
| 23 | ♂ | 1900 | 5.41 | 6.15 | 6.48 | 9.65 | 11.10 | 7.86 | 21.45 | 15.3 | 4.1 | Figures on lung, kidney, brain, and heart are on combined organs of Rabbits 23 and 27. |
| 24 | ♀ | 1850 | 5.78 | 5.82 | 8.71 | 9.51 | | 8.79 | 24.64 | 15.3 | 4.2 | Figures on lung, kidney, brain, and heart are on combined organs of Rabbits 24 and 26. |
| 25 | ♂ | 1950 | 6.47 | 6.27 | 8.50 | 8.10 | 9.26 | 9.38 | 15.63 | 13.7 | 4.2 | |
| 26 | ♀ | 1700 | 6.22 | 6.33 | | | | | 16.65 | 16.2 | 6.6 | |
| 32 | ♀ | 1100 | 5.48 | 8.42 | | | | 8.68 | 21.63 | 12.2 | | Rabbits 32–38 inclusive used as controls. |
| 33 | ♀ | 1200 | 5.97 | 7.10 | 7.65 | 10.05 | 7.04 | | 17.7 | 12.0 | 6.6 | Figures on lung, kidney, brain, and heart are on combined organs of Rabbits 32 and 33. |
| 34 | ♂ | 1200 | 4.58 | 9.03 | | | | | 20.7 | 12.1 | 7.0 | |
| 35 | ♀ | 1200 | 5.12 | 7.22 | 5.91 | 8.46 | 4.53 | | | 10.3 | 6.4 | Figures on lung, kidney, and brain are on combined organs of Rabbits 34 and 35. |
| 36 | ♂ | 1500 | 6.87 | 6.13 | | | | | 20.9 | 13.5 | 6.6 | |
| 37 | ♂ | 1400 | 6.45 | 9.33 | 6.03 | 7.02 | 5.62 | | 17.57 | 11.7 | 6.0 | Figures on lung, kidney, and brain are on combined organs of Rabbits 36 and 37. |
| 38 | ♀ | 900 | 6.17 | 10.26 | 9.41 | 10.22 | 6.46 | | 16.25 | 11.7 | 6.0 | |

TABLE III.

*Average of Results on Rabbit Tissues.*

| | | Calcium per 100 gm. | | | | | | Per 100 cc. serum. | |
|---|---|---|---|---|---|---|---|---|---|
| | | Muscle. | Liver. | Lung. | Kidney. | Brain. | Bone. | Ca | Inorganic P. |
| | | *mg.* | *mg* | *mg.* | *mg.* | *mg.* | *m.* | *mg.* | *mg.* |
| 5 rabbits given $CaCl_2$. | Max. | 9.25 | 9.15 | | | | 13.2 | 15.3 | 4.1 |
| | Min. | 6.00 | 7.71 | | | | 11.6 | 12.6 | 3.7 |
| | Average. | 7.40 | 8.70 | | | | 12.48 | 14.8 | 3.9 |
| 8 rabbits given $CaCl_2$ and irradiated. | Max. | 6.60 | 8.74 | 10.82 | 9.65 | 11.10 | 24.68 | 16.0 | 4.2 |
| | Min. | 5.41 | 5.82 | 6.62 | 6.84 | 5.63 | 16.20 | 13.5 | 3.3 |
| | Average. | 6.1 | 6.67 | 8.22 | 8.56 | 8.33 | 18.69 | 14.7 | 3.8 |
| 7 rabbits given Ca lactate. | Max. | 9.32 | 12.95 | 11.51 | 11.88 | | 13.8 | | |
| | Min. | 4.58 | 5.31 | 10.75 | 8.05 | | 12.5 | | |
| | Average. | 6.74 | 7.68 | 11.3 | 9.96 | | 12.98 | | |
| 8 rabbits given Ca lactate and irradiated. | Max. | 8.58 | 8.25 | 16.60 | 11.50 | 11.8 | 24.35 | 16.0 | 5.64 |
| | Min. | 5.13 | 5.77 | 7.08 | 8.10 | 8.21 | 19.8 | 15.1 | 5.08 |
| | Average | 6.97 | 6.83 | 11.19 | 9.64 | 7.20 | 21.8 | 15.38 | 5.25 |
| 6 control rabbits not irradiated. | Max. | 8.37 | 11.6 | | | | 16.15 | 15.6 | 4.4 |
| | Min. | 3.99 | 5.83 | | | | 12.31 | 14.1 | 5.1 |
| | Average. | 6.10 | 7.51 | | | | 14.22 | 14.99 | 4.92 |
| 7 control rabbits irradiated. | Max. | 6.87 | 10.26 | 9.41 | 10.22 | 7.04 | 21.63 | 13.5 | 7.0 |
| | Min. | 4.58 | 6.13 | 5.91 | 7.02 | 4.53 | 16.25 | 11.7 | 6.0 |
| | Average. | 5.81 | 8.21 | 7.25 | 8.92 | 5.92 | 20.3 | 11.94 | 6.52 |

by the animals, was given in small porcelain evaporating dishes so that it was easily possible to observe whether the entire amount had been consumed. The control animals received daily a similar amount of carrots, to which no calcium salts had been added.

In our first experiment seventeen rabbits were used; some of these received daily 1.0 gm. of calcium lactate (equivalent to 0.18 gm. calcium) for a period of 34 days, five received 0.5 gm. of calcium chloride (equivalent to 0.18 gm. calcium) for 49 days, and six animals used as controls were kept in the same animal rooms and fed the same ration of oats, hay, and carrots for a period of 35 days.

In our second experiment twenty-three rabbits were used. Eight of these animals were given 1.0 gm. of calcium lactate daily for 31 days, eight received 0.5 gm. of calcium chloride for 38 days, and seven animals used as controls were killed after 43 days. All of this group of animals received daily exposure for 30 minutes to the light from a Cooper-Hewitt mercury tungsten arc which was run on a direct current of 120 volts and 5 amperes.

During exposure the animals, whose eyes were protected by hoods, were placed in groups of four in such a position that they were about 18 inches from the quartz tube. These rabbits were not shaved and were of various shades of white, brown, and gray.

At the end of the experimental period the animals were killed by a blow on the head, and samples of blood and tissue immediately taken for analysis. In every case the bony tissues were represented by one entire femur. The viscera and muscles were rapidly removed, dissected free of fat and connective tissue, ground in a meat grinder, and stored in weighing bottles provided with well fitting glass stoppers. The determination of calcium in tissues was carried on by the method described in the preceding paper. Calcium in serum was determined by the method of Kramer and Tisdall (7) and inorganic phosphate by the procedure of Briggs (8).

The results are given in Tables I to III.

In Table I we have collected the results obtained on the non-irradiated rabbits, while Table II contains the values on the irradiated animals. In Table III we have tabulated the maxi-

| 7 control rabbits irradiated. | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Max. | 6.87 | 10.26 | 9.41 | 10.22 | 7.04 | 21.63 | 13.5 | 7.0 |
| | Min. | 4.58 | 6.13 | 5.91 | 7.02 | 4.53 | 16.25 | 11.7 | 6.0 |
| | Average. | 5.81 | 8.21 | 7.25 | 8.92 | 5.92 | 20.3 | 11.94 | 6.52 |

mum, minimum, and average figures for both series of experiments.

A consideration of the average figures collected in Table III would seem to show that the administration by mouth of calcium chloride or calcium lactate with or without exposure to ultra-violet light is without effect on the concentration of calcium in the tissues, a finding which is in accord with the results of Heubner and Rona on dogs to which calcium chloride was administered by subcutaneous injection. The high content of calcium in the bones of all of the animals exposed to ultra-violet light, with or without calcium treatment, is, however, marked.

The figures for calcium and inorganic phosphate of the blood serum are included to complete the record. We felt a considerable degree of distrust of the serum calcium figures when they were obtained on account of the general high level and the more or less definite variations in the concentrations in each batch of animals. Determinations were therefore made on a number of specimens of serum after ashing, but with results which checked well with those obtained by the Kramer-Tisdall method. Furthermore, samples of serum from normal human blood when run in series with these rabbit sera gave results well within normal limits.

We feel that these results may perhaps be explained by the observations recently published by Grant and Gates (9) regarding the fluctuations in the level of the calcium and inorganic phosphates of the blood of rabbits confined in cages over considerable periods. The results reported by these investigations furnish knowledge of an experimental factor which must henceforth be taken into account in the interpretation of the results of work of this kind.

The figures given for inorganic phosphate in serum showed the highest level in the group of animals irradiated without the administration of calcium salts, and the lowest in those animals receiving calcium chloride either with or without irradiation. There is no apparent relation between the levels of calcium and of inorganic phosphates in these sera.

The effect of exposure to ultra-violet light in raising the inorganic phosphate or calcium in the blood of children suffering from rickets (10) has been repeatedly demonstrated, but data

regarding the effect of irradiation on normal animals are scanty; the results published by Grant and Gates and by Pincussen and Markineos (11) indicate that small effect, if any, is produced in either of these constituents of the serum when normal animals are subjected to irradiation.

SUMMARY.

Rabbits receiving a daily dose of calcium chloride or calcium lactate showed no higher calcium content of the tissues than did the control animals. Daily exposure to ultra-violet light from a mercury tungsten arc was also without effect on the calcium content of the tissues and serum.

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# THE CHEMICAL INVESTIGATIONS OF CORPUS LUTEUM.

## IV. THE ACETONE-SOLUBLE FAT.

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(Received for publication, October 15, 1925.)

Most modern investigators agree that the acetone extract of the ovary contains the hormone which governs the phenomena of estrus. Evidence seems to be quite conclusive also that this growth-stimulating and regulating factor is contained in the unsaponifiable fraction. This dramatic search for the so called active principle has more or less overshadowed other important questions as to the physiological action of the ovary. For instance, what is the chemical nature of the neutral fat of corpus luteum? Are the fatty acids of this fat similar in nature to the fatty acids incorporated in the molecules of the different phosphatides? Are these phosphatides specific in their chemical structure or are they closely related to the phosphatides elaborated in other glandular structures, indicating a common function?

Our experimental investigation of the fatty acids in the neutral fat of corpus luteum shows that there are present the same fatty acids that have been shown by Levene and others to be present in lecithin and cephalin isolated from other tissues. This would indicate that the neutral fat functions more or less as a menstruum for the production of these non-specific lipoids. We would expect therefore that the hydrolysis of the lecithin and cephalin from corpus luteum would give fatty acids similar to those obtained from the phosphatides isolated from other tissues. This would remove the possibility that there was anything specific in the physiology of these phosphatides and focus more clearly attention as to just

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what is the exact function of these phosphatides in the metabolic processes.

In the neutral fat of corpus luteum we have shown the presence of approximately 8 per cent of arachidonic acid. Wesson[1] has suggested the possibility that arachidonic acid is an intermediate product in the metabolism of at least part of the fatty acids which contain fewer than 20 carbon atoms. Of great importance in this question of the function of arachidonic acid in metabolism, is our finding of relatively large quantities (4.8 per cent) of a hexa-unsaturated acid of the $C_{20}$ series. This also brings up the question as to the relation of the phosphatides, such as lecithin and cephalin, to the utilization by the body of this arachidonic acid. It is quite possible that the production of arachidonic acid regulates more or less the chemical physical properties of these phosphatides and thus plays a very important part in the metabolic processes. This question will be attacked later by carrying out this same sort of chemical investigation on corpora lutea collected at different known stages of pregnancy and attempting to correlate this chemical information with the known physiological conditions represented. Before this was done, however, we considered it of importance to carry out this preliminary study on the commercially important, easily procurable, pharmaceutical corpus luteum.

## EXPERIMENTAL.

The sample of corpus luteum used in the following experiments represented carefully collected, hand-dissected material, dried immediately after collection in a high vacuum and below 40°C. in the commercial way. The dried corpus luteum represents approximately 6 parts of the fresh corpus luteum substance. It was representative of the material found on the pharmaceutical market in that it had been taken from such a large number of cattle as to represent a good average sample. It contained 4.5 per cent moisture and 5.4 per cent ash.

The material was ground to pass through a No. 20 sieve. 5.5 kilos of this dried ground tissue were exhaustively extracted with five 5 liter portions of acetone.

The combined acetone extracts were concentrated to about 1

[1] Wesson, L. G., *J. Biol. Chem.*, 1925, lxv, 235.

liter. This solution upon standing deposited some crystalline material which was shown[2] to consist of cholesteryl palmitate, potassium stearate, fatty acids, phosphatides,[3] and a mixture of the amino acids[4] leucine, isoleucine, and valine.

The acetone filtrate and washings from the above described crystalline material were evaporated to dryness and the residue dissolved in 1 liter of pure acetone. On standing in the cold for some time material separated which when dried *in vacuo* weighed 9.025 gm. Examination of this material indicated that it consisted of cholesteryl palmitate, a potassium soap, possibly sphingomyelin, and an alcohol-soluble phosphatide similar to lecithin.[3]

The acetone filtrate and washings from the above described material were concentrated and the oily residue dissolved in 2 liters of absolute alcohol. Upon standing in the cold this solution gradually deposited some white material. This was filtered off, washed, and dried *in vacuo*. This material weighed 5.66 gm. and investigation showed it to consist of cholesteryl palmitate mixed with the fat, tristearin.[3]

The filtrate from this fatty material gave nothing more of a crystalline nature when held in the cold at various concentrations. The entire alcohol solution measuring 2 liters was then precipitated completely with alcoholic cadmium chloride solution. A white flocculent precipitate of the lecithin cadmium chloride[3] compound was obtained. The alcoholic mother liquor from this precipitation was taken to dryness and the oily residue dissolved in ether, filtered from the insoluble cadmium chloride, and the ether solution concentrated to a volume of 2 liters.

The ether solution of the fat was extracted with water and 1 per cent sulfuric acid to remove water-soluble nitrogen bases and inorganic salts. Water extracted 108 gm. of solid material containing 2.27 per cent N, 0.32 per cent P, and 29 per cent cadmium (48 per cent cadmium chloride). The 1 per cent sulfuric acid extract was small and contained a total of 0.11 gm. of nitrogen and 0.11 gm. of phosphorus.

The purified fat weighed 720 gm. and contained 0.36 per cent N and 0.24 per cent P. For preliminary analysis, 35 gm. of the fat

[2] Hart, M. C., and Heyl, F. W., *J. Am. Pharm. Assn.*, 1924, xiii, 17.
[3] Hart, M. C., and Heyl, F. W., *J. Biol. Chem.*, 1925, lxvi, 639.
[4] Hart, M. C., and Heyl, F. W., *J. Am. Pharm. Assn.*, 1925, xiv. 770.

were dried to constant weight and the following determinations made. Where possible official methods as given in Bulletin 107, United States Department of Agriculture, were used, otherwise Lewkowitsch[5] was followed:

| | |
|---|---|
| Specific gravity $\frac{100°}{100°}$C. | 0.9031 |
| Iodine No. (Hanus) | 66 |
| Acid value | 78 |
| Saponification value | 154 |
| Unsaponifiable | 15.6 per cent |
| Reichert Wollny value | 0.76 |
| Cholesterol[6] | 10.2 per cent |
| Acetyl value | 36 |

700 gm. of the fat in ether solution were extracted with 10 per cent potassium hydroxide to remove the free fatty acids. The ether solution of neutral fat was then taken to dryness and saponified by boiling for 8 hours with 20 per cent alcoholic potash. Most of the alcohol was removed by distillation,[7] and the residual soap dissolved in water. The alkaline soap solution was then extracted repeatedly with ether to remove the unsaponifiable material. The fatty acids from the saponification of the neutral fat were obtained by decomposing the soap solutions with 20 per cent hydrochloric acid and extracting the liberated fatty acids with ether.

By this procedure, the 700 gm. of fat were separated into the following three fractions:

1. 254 gm. free fatty acids.
2. 270 " fatty acids from neutral fat.
3. 105 " unsaponifiable material.

The unsaponifiable material was crystallized from alcohol to remove cholesterol. 58 gm. of cholesterol were obtained melting at 143–144°C. The alcoholic mother liquor from the cholesterol

[5] Lewkowitsch, J., Chemical technology and analysis of oils, fats and waxes, London, 6th edition, 1921, i.

[6] Method of MacArthur (MacArthur, C. G., *J. Am. Chem. Soc.*, 1919, xli, 1232) gave 66.3 per cent cholesterol in the unsaponifiable fraction, or 10.2 per cent in the total fat.

[7] All distillations were conducted at reduced pressure in an atmosphere of nitrogen.

was ampouled in an atmosphere of nitrogen and reserved for future physiological and chemical examination.

*The Free Fatty Acids.*

The 254 gm. of free fatty acids in ether solution were extracted with varying strengths of alkaline solutions. The fatty acids were obtained from the alkaline extracts by acidifying with hydrochloric acid and extracting the liberated fatty acids with ether. The following fractions were obtained:

| | | | |
|---|---|---|---|
| 10 per cent | ammonium carbonate | extract, | 16 gm. acids. |
| 10 " " | sodium " | " | 236 " " |
| 10 " " | potassium hydroxide | " | 2 " " |

*The Ammonium Carbonate Extract of the Free Fatty Acids.*

This fraction consists of 16 gm. of a dark brown oil having a strong odor of volatile acids. Nothing crystalline was obtained upon allowing the alcoholic solution to stand at various concentrations in the ice box.

The oil was mixed with 10 per cent sulfuric acid and steam distilled. The total volatile acids in the 700 cc. distillate were equivalent to 3.3 cc. of normal acid. The solution was neutralized and concentrated to 100 cc. The soap solution was acidified, the volatile acids extracted with ether, and dried over anhydrous sodium sulfate. The ether was removed *in vacuo*, the residual acid dissolved in 5 per cent ammonia solution, and the excess ammonia boiled off. The solution was precipitated with silver nitrate, the precipitate filtered, washed, and dried. The silver salt was analyzed for silver.

*Analysis.* 0.0669 gm. substance: Ag 0.0267.
Calculated for $C_8H_{15}O_2Ag$. Ag 42.8.
" " $C_{10}H_{19}O_2Ag$. " 38.5.
Found. Ag 39.9 per cent.

These results suggest that the volatile acids consist of a mixture of caprylic and capric acids.

The non-volatile acids from the steam distillation were extracted with ether, washed free from sulfuric acid, and dried over anhydrous sodium sulfate. The ether was removed and the residual acids distilled *in vacuo*. The distillate, consisting of 6.8 gm. of a

partially solid oil, distilled at 170–200°C. at a pressure of 3 to 4 mm. of mercury. The tarry residue in the distilling flask was not further examined. The distilled acids crystallized from 40 cc. of 95 per cent alcohol yielded 0.65 gm. of palmitic acid melting at 58–61°.

*Analysis.* 0.1561 gm. substance: $CO_2$ 0.4292, $H_2O$ 0.1782.
Calculated for $C_{16}H_{32}O_2$. C 75.0, H 12.5.
Found. C 75.0, H 12.8.

The mother liquor from these crystals was separated into the saturated and unsaturated acids by the lead salt-ether process.[8] 1.8 gm. of saturated acids and 3.5 gm. of unsaturated acids were obtained.

The saturated acids were crystallized from alcohol and two fractions obtained.

*Fraction 1.* (0.39 gm.) m.p. 55–56°C.
*Analysis.* 0.1490 gm. substance: $CO_2$ 0.4099, $H_2O$ 0.1693.
Calculated for $C_{16}H_{32}O_2$. C 75.0, H 12.5.
Found. C 75.0, H 12.7.

*Fraction 2.* (0.92 gm.) m.p. 52–54°C.
*Analysis.* 0.1601 gm. substance: $CO_2$ 0.4388, $H_2O$ 0.1804.
Calculated for $C_{16}H_{32}O_2$. C 75.0, H 12.5.
Found. C 74.8, H 12.6.

Both of these fractions analyze for palmitic acid indicating that the saturated acids of the ammonium carbonate extract consist entirely of palmitic acid. Myristic acid is not present in this fraction.

The unsaturated acids (3.5 gm.) from the lead salt separation consisted of a brown oil having an iodine number of 151 and a neutralization value of 174. As the neutralization value is abnormally low probably due to the condensation of the unsaturated acids, this fraction was reduced with hydrogen and colloidal palladium by the method of Paal. The saturated acids so obtained were crystallized from alcohol. 2.0 gm. of unsaturated acids yielded 1.27 gm. of crystalline acids melting at 59–60°C.

[8] Lewkowitsch, J., Chemical technology and analysis of oils, fats and waxes, London, 6th edition, 1921, i, 556.

*Analysis.* 0.1495 gm. substance: $CO_2$ 0.4179, $H_2O$ 0.1751.
Calculated for $C_{18}H_{36}O_2$. C 76.0, H 12.7.
" " $C_{20}H_{40}O_2$. " 76.9, " 12.8.
Found. C 76.3, H 13.1.

These analytical results indicate a mixture of stearic and arachidic acids. The unsaturated acids, therefore, consist of a mixture of $C_{18}$ and $C_{20}$ acids. The iodine number of the unsaturated acids (151) and the analysis of the reduced acid indicate that these acids consist of a mixture of 70 per cent oleic ($C_{18}H_{34}O_2$) and 30 per cent arachidonic ($C_{20}H_{32}O_2$) acids. Arachidonic acid was later isolated from the unsaturated acids of the sodium carbonate extracts.

*The Sodium Carbonate Extract of the Free Fatty Acids.*

The 236 gm. of acids extracted by 10 per cent sodium carbonate were dissolved in 95 per cent alcohol and cooled in the ice box. The crystalline acids which separated were recrystallized from 95 per cent alcohol. 40 gm. of crystals melting at 53–55°C. were obtained. As these acids were similar to the fatty acids from the neutral fat, they were joined with these and studied together.

The alcoholic mother liquor from the above crystallization yielded 182 gm. of fatty acids. These acids were distilled *in vacuo*. The distillate consisted of 155 gm. of a clear yellow oil which partially solidified upon cooling. 22 gm. of these acids were separated into the saturated and unsaturated acids by the lead salt-ether process. This separation yielded 12.1 gm. of liquid acids having an iodine number of 165, and 8 gm. of solid acids having an iodine number of 80. This method of separation was unsatisfactory because of the large amount of unsaturated acids contaminating the saturated fraction. The remainder of the acids (133 gm.) were separated by the barium-lead salt process.[9]

This separation is based upon the fact that the barium salts of oleic acid and also of the saturated acids are insoluble in a mixture of benzene and ether. The barium salts of the more unsaturated acids are soluble in this mixture. Oleic acid is separated from the saturated acids by the solubility of its lead salt in ether, the lead salts of the saturated acids being insoluble in ether.

[9] Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1922, li, 509.

By this means the acids were separated into the following three fractions.

1. 90 gm. unsaturated acids, iodine No. 165.
2. 11 " intermediate fraction, iodine No. 128.
3. 30 " saturated acids, iodine No. 21.

These fractions were later joined for analysis to like fractions of acids from the neutral fat.

*The Potassium Hydroxide Extract.*

These acids (2 gm.) were crystallized from alcohol. A small amount of crystalline acids was obtained which melted at 55°C. This appeared to be a similar mixture to that obtained from the sodium carbonate extract of free acids, and was not further examined.

*The Fatty Acids of the Neutral Fat.*

These acids weighing 270 gm. were dissolved in ether and extracted with varying strengths of alkaline solutions. The fatty acids were recovered from the alkaline extracts in the usual manner. In this case, 10 per cent ammonium carbonate and 10 per cent potassium hydroxide extracted only small amounts of acids from which nothing crystalline could be obtained. Most of these acids (267 gm.) were extracted by 10 per cent sodium carbonate.

The acids of the sodium carbonate extract were crystallized from 95 per cent alcohol. The crystalline acids which separated, after recrystallization from alcohol, weighed 34 gm. and melted fairly sharply at 54°C. A mixed melting point with pure myristic acid indicated that this was not myristic acid. These solid acids were later examined together with the other saturated acids of the fat.

The alcoholic mother liquor from the above crystallization yielded 233 gm. of acids. These were distilled *in vacuo*. The distillate consisted of 213 gm. of a yellow oil which partially solidified at room temperature, and had an iodine number of 127. These acids were separated into three fractions by the barium-lead salt process. The following fractions were obtained.

1. 146 gm. unsaturated acids, iodine No. 169.
2. 20 " intermediate fraction, iodine No. 120.
3. 45 " saturated acids, iodine No. 21.

These fractions had similar properties to like fractions obtained from the free fatty acids. Before further analytical work, like fractions of the free acids and those from the neutral fat were joined. The saturated acids obtained by the barium-lead salt separation were recrystallized from alcohol and joined to the crystalline acids which separated from the mixed acids before distillation.

TABLE I.

*Distillation of Unsaturated Acids.*

| Fraction. | Weight. | Boiling point. | Pressure. | Iodine No. | Neutralization value. | Molecular weight. |
|---|---|---|---|---|---|---|
| | *gm.* | *°C.* | *mm. Hg* | | | |
| 1 | 10.1 | To 180 | 1.2–1.4 | 106 | 191.5 | 293 |
| 2 | 20.2 | 180–185 | 1.5 | 117 | 193.0 | 291 |
| 3 | 32.2 | 185–190 | 1.4–1.5 | 140 | 193.7 | 290 |
| 4 | 36.4 | 190–195 | 1.5 | 159 | 188.6 | 297 |
| 5 | 34.9 | 195–200 | 1.5 | 189 | 185.4 | 302 |
| 6 | 22.3 | 200–205 | 1.4–1.5 | 224 | 178.0 | 315 |
| 7 | 11.4 | 205–207 | 1.5 | 251 | 171.4 | 327 |
| 8 | 7.0 | 207–210 | 1.5 | 274 | 166.9 | 336 |
| Residue..... | ..... | ..... | ..... | 199 | | |

*Examination of the Highly Unsaturated Acids.*

These acids consisted of 236 gm. having an iodine number of 169. The iodine number indicated that highly unsaturated acids are present.

209 gm. of these acids were fractionally distilled at a pressure of 1.5 mm. of mercury. Five fractions and a residue were obtained. These were refractionated into eight fractions. Neutralization values and iodine numbers of the fractions were determined and their mean molecular weights calculated. These results are summarized in Table I.

From fractions 1 and 2, there separated a small amount of solid fatty acids which had the characteristics of the saturated acids of the fat and was not further examined.

Each of the eight fractions from the above distillation was

dissolved in dry ether, cooled to 0°C., and saturated with bromine. After standing overnight in the ice box, the ether-insoluble bromides were separated by centrifuging, washed free from bromine with cold ether, dried, and weighed. Excess of bromine was removed from the ether-soluble bromides by emulsification with sodium thiosulfate solution.

The ether-soluble bromides were then dried, dissolved in dry ethyl alcohol, and debrominated by boiling for 3 hours during which a stream of dry hydrogen chloride was passed through the solution, and zinc dust added in small quantities. The alcoholic solution was diluted with a large volume of water and the acid extracted with ether. The acid so obtained (which also contains

TABLE II.

*Bromination of Unsaturated Acids.*

| Fraction. | Acid taken. | Ether-insoluble bromides. | Br in bromides (Carius). | Tetrabromide. | Dibromide | Acid from dibromide. | Iodine No. Acid from dibromide. |
|---|---|---|---|---|---|---|---|
| | *gm.* | *gm.* | *per cent* | *gm* | *gm.* | *gm.* | |
| 1 | 7.0 | 0.78 | 63.5 | 0.39 | 10.7 | 6.15 | 96 |
| 2 | 10.3 | 1.15 | 61.9 | 0.69 | 14.3 | 8.07 | 103 |
| 3 | 11.4 | 2.50 | 65.4 | 0.06 | 18.0 | 9.53 | 116 |
| 4 | 13.7 | 4.06 | 64.7 | 0.06 | 22.0 | 11.3 | 128 |
| 5 | 13.9 | 8.0 | 64.5 | 0.21 | 17.1 | 8.30 | 135 |
| 6 | 14.0 | 13.0 | 64.1 | 0.14 | 18.3 | 8.50 | 151 |
| 7 | 9.9 | 11.3 | 65.0 | 0.20 | 12.4 | 5.32 | 161 |
| 8 | 5.7 | 6.55 | 66.4 | 0.35 | 7.8 | 2.78 | 173 |

ethyl ester) was again brominated. In this way a further quantity of ether-insoluble bromides was obtained. These bromides were weighed but were not joined to the first crop because they contained the bromides of the ethyl esters which were formed during the debromination.

The ethereal filtrate from the second crop of insoluble bromides was emulsified with sodium thiosulfate to remove excess bromine, dried over anhydrous sodium sulfate, and the ether removed. The bromides were then taken up in petroleum ether and the insoluble tetrabromide fraction separated by centrifuging. The bromides soluble in pretoleum ether were dried and weighed. This dibromide fraction was then debrominated with alcoholic

hydrogen chloride and zinc. The ethyl ester so obtained was saponified, the soap decomposed with dilute sulfuric acid, and the fatty acid extracted with ether, the ether solution dried, and the ether removed. The iodine number was determined for each fraction. The results of the bromination are given in Table II.

*The Ether-Insoluble Bromides.*

This substance consisted of material which in fractions 1 to 6 was a pure white, easily powdered substance. The bromides from fractions 7 and 8 became gray when dried *in vacuo* at 60°C. These bromides blackened and decomposed at 225–230°C. The bromine content (Table II) indicates that these fractions consist of a mixture of arachidonic octobromide ($C_{20}H_{32}O_2Br_8$) and a hexabromide.

18.7 gm. of the ether-insoluble bromides from fractions 4, 5, and 6 were extracted in a Soxhlet with benzene. The benzene-insoluble bromide (6.1 gm.) was washed with large quantities of cold dry ether. The resulting product (5.7 gm.) blackened and decomposed at 237–238°C. This was analyzed for bromine.

*Analysis.* 0.1465 gm. substance: AgBr 0.2321.
Calculated for $C_{20}H_{32}O_2Br_8$. Br 67.8.
Found. Br 67.4.

These results indicate that the product is nearly pure arachidonic octobromide.

5.5 gm. of the purified octobromide were debrominated with alcoholic hydrogen chloride and zinc dust by the method of Wesson.[10] The resulting ethyl ester was saponified and the acid obtained in the usual manner. 1.1 gm. of unsaturated acid were obtained.

0.0920 gm. acid absorbed 0.2846 gm. iodine. Iodine No. 309.
Calculated for $C_{20}H_{32}O_2$. Iodine No. 335.

0.78 gm. of this acid was reduced in alcoholic solution with hydrogen and colloidal palladium. 0.68 gm. of a crystalline acid was obtained melting at 65–67°C. After two crystallizations from 95 per cent alcohol, 0.43 gm. of a pure white crystalline acid

[10] Wesson, L. G., *J. Biol. Chem.*, 1925, lx, 183.

was obtained which melted at 68–69°C. Further crystallization failed to raise the melting point. This acid mixed with stearic (m.p. 69) melted at 61–62°C., indicating that it is not stearic acid.

*Analysis.* 0.1443 gm. substance: $CO_2$ 0.4067, $H_2O$ 0.1670.
Calculated for $C_{20}H_{40}O_2$. C 76.9, H 12.9.
Found. C 76.9, H 12.9.

The reduced acid is pure arachidic acid, thus proving that the unsaturated acid in the fat is arachidonic acid, $C_{20}H_{32}O_2$.

The benzene extract upon cooling deposited 7.5 gm. of a bromide which blackened at 220–225°C. This material was crystallized from benzene into two fractions, both of which blackened at 218–222°C.

*Fraction 1.* 5.38 gm.
*Analysis.* 0.1479 gm. substance: AgBr 0.2242.
Found. Br 64.5.
*Fraction 2.* 0.63 gm.
*Analysis.* 0.1435 gm. substance: AgBr 0.2168.
Found. Br 64.3.
Calculated for $C_{20}H_{32}O_2Br_8$. Br 67.8.
" " $C_{20}H_{34}O_2Br_6$. " 61.0.
" " $C_{18}H_{30}O_2Br_6$. " 63.3.

These analytical results indicated a hexabromide, but the blackening of these bromides at 220°C. instead of melting at 181°C. indicated that it is not linolenic hexabromide ($C_{18}H_{30}O_2Br_6$).

Fractions 1 and 2 were joined, suspended in absolute ethyl alcohol, and debrominated with zinc dust and dry hydrogen chloride. The ethyl ester so obtained was saponified, the soap decomposed with dilute sulfuric acid, and the liberated acid extracted with ether. 5.7 gm. of bromides yielded 1.95 gm. of acid having an iodine number of 270. (0.1058 gm. absorbed 0.2857 gm. iodine.)

1.8 gm. of this acid were reduced with hydrogen and colloidal palladium. After one crystallization, 1.5 gm. of acid were obtained melting at 67–68°C. This was recrystallized from alcohol into two fractions and analyzed.

*Analyses.*
*Fraction 1.* 1.2 gm., m. p. 68–69°C.
0.1544 gm. substance: $CO_2$ 0.4359, $H_2O$ 0.1818.
Found. C 77.0, H 13.2.

*Fraction 2.* 0.2 gm., m. p. 66–67°C.
0.1537 gm. substance: $CO_2$ 0.4308, $H_2O$ 0.1781.
Found. C 76.5, H 13.0.
Calculated for $C_{20}H_{40}O_2$. C 76.9, H 12.9.
" " $C_{18}H_{36}O_2$. " 76.0, " 12.8.

These analytical results indicate that fraction 1 is pure arachidic acid and fraction 2 is impure arachidic acid. Since most of the acid is represented in fraction 1, we conclude that practically all of the reduced acid is arachidic, proving that the unsaturated acid in the fat is of the $C_{20}$ series. The iodine number of the unsaturated acid (270) and also the bromine content (64.5 per cent) of the benzene-soluble bromides from which this acid was obtained, indicate that it is a mixture of about equal parts of arachidonic acid ($C_{20}H_{32}O_2$) and a $C_{20}$ acid with three double bonds having the formula $C_{20}H_{34}O_2$. If this unsaturated acid were a mixture of arachidonic acid and linolenic acid ($C_{18}H_{30}O_2$), the reduction product would have been 73 per cent stearic acid and 27 per cent arachidic, instead of nearly all arachidic, as we found. These analytical results prove the presence of an unsaturated acid in the fat having the formula $C_{20}H_{34}O_2$.

*The Petroleum Ether-Insoluble Bromides.*

The amounts of petroleum ether-insoluble bromides (Table II) were very small in all of the fractions. Those from fractions 1 and 2, consisting of 1.08 gm., were taken up in a small amount of cold dry ether and filtered from the insoluble higher bromides. The ether solution was taken to dryness and the residue repeatedly extracted with petroleum ether. The insoluble residue (0.7 gm.) was crystallized in two fractions from a minimum amount of dry ether. Both fractions melted at 110–112°C. Linolic tetrabromide melts at 113–114°C.

*Analyses.*
*Fraction 1.* 0.1124 gm. substance: AgBr 0.1410.
Found. Br 53.4.
*Fraction 2.* 0.1709 gm. substance: AgBr 0.2180.
Found. Br 54.3.
Calculated for $C_{18}H_{32}O_2Br_4$. Br 53.3.

These analytical results indicate that the petroleum ether-insoluble bromide is linolic tetrabromide.

*The Acids from the Petroleum Ether-Soluble Bromides.*

These acids obtained by debrominating the liquid bromides (Table II) represent the largest part of the unsaturated acids. The iodine values, varying from 96 in fraction 1 to 173 in fraction 8, indicate that acids more unsaturated than oleic are present. Rollett[11] has shown that when linolic acid is treated with bromine, only about 50 per cent of it is converted into a crystalline, petroleum ether-insoluble bromide, the other 50 per cent appearing as

TABLE III.

*Analysis of Acids from the Liquid Bromides.*

| Fraction. | Unsaturated acid taken. | Iodine value. | Saturated acids after crystallization. | Melting point. | Substance. | $CO_2$ | $H_2O$ | C | H | Neutralization value. |
|---|---|---|---|---|---|---|---|---|---|---|
| | *gm.* | | *gm.* | *°C.* | *gm.* | *gm.* | *gm.* | *per cent* | *per cent* | |
| 1 | 3.0 | 96 | 1.38 | 65 | 0.1434 | 0.3978 | 0.1643 | 75.7 | 12.8 | 197 |
| 2 | 3.0 | 103 | 1.75 | 66–67 | 0.1374 | 0.3828 | 0.1589 | 76.0 | 12.9 | 197 |
| 3 | 3.0 | 116 | 1.77 | 66–67 | 0.1508 | 0.4197 | 0.1721 | 75.9 | 12.8 | 196 |
| 4 | 3.0 | 128 | 1.86 | 64–65 | 0.1545 | 0.4293 | 0.1760 | 75.8 | 12.7 | 195 |
| 5 | 3.0 | 135 | 1.72 | 63–64 | 0.1585 | 0.4414 | 0.1821 | 76.0 | 12.9 | 191 |
| 6 | 3.0 | 151 | 1.86 | 62 | 0.1528 | 0.4276 | 0.1750 | 76.3 | 12.8 | 186 |
| 7 | 3.0 | 161 | 1.17 | 65–66 | 0.1438 | 0.4043 | 0.1672 | 76.7 | 13.0 | 181 |
| 8 | 2.6 | 173 | 0.73 | 68–69 | 0.1537 | 0.4310 | 0.1772 | 76.5 | 12.9 | 169 |
| Calculated for | | | | | | | | | | |
| $C_{18}H_{36}O_2$ | | | | 69 | | | | 76.0 | 12.7 | 197.5 |
| $C_{20}H_{40}O_2$ | | | | 77 | | | | 76.9 | 12.9 | 179.8 |

an oily petroleum ether-soluble bromide, which when debrominated yields linolic acid. Therefore, it seemed likely that linolic acid might be present in these fractions.

These acids were reduced with hydrogen and colloidal palladium and the resulting saturated acids crystallized three times from 95 per cent alcohol. These crystalline acids were analyzed. The results are given in Table III.

These analytical results show that fractions 1 to 5 consist of

[11] Rollett, A., *Z. physiol. Chem.*, 1909, lxii, 410.

unsaturated acids of the $C_{18}$ series. Fractions 6, 7, and 8 contain increasing amounts of acids of the $C_{20}$ series. The iodine values of fractions 6, 7, and 8 also indicate that some arachidonic acid is present which did not separate as an ether-insoluble brom-derivative.

To get further information about these unsaturated acids from the dibromide fractions, the oxidation products were studied. 6 gm. of acid obtained from the reduction of the dibromide of fraction 3 (iodine No. 116) were oxidized in alkaline solution with potassium permanganate.[12] 1.8 gm. of water-insoluble oxidation products were obtained. Of this material, ether dissolved 1.6 gm. Upon concentrating and cooling the ether solution, the dihydroxystearic acid crystallized out. This was recrystallized from ether and boiled three times with 200 cc. of water. The water-insoluble residue was crystallized from 95 per cent alcohol. 0.568 gm. of a white crystalline acid was obtained which melted at 129–130°C.

*Analysis.* 0.1403 gm. substance: $CO_2$ 0.3499, $H_2O$ 0.1443.
Calculated for $C_{18}H_{36}O_4$. C 68.3, H 11.4.
Found. C 68.0, H 11.5.

This is dihydroxystearic acid indicating the presence of oleic acid in this fraction.

The ether-insoluble, sativic fraction (0.2 gm.) was crystallized from boiling water, extracted with ether, and recrystallized from 95 per cent alcohol. 0.09 gm. of acid melting at 155–162°C. was obtained. In order to obtain more of this material, 8 gm. of acid from the reduction of the dibromide of fraction 4 (iodine No. 128) were oxidized and the sativic fraction obtained. 0.22 gm. of an acid melting at 155–160°C. was obtained. This material was repeatedly extracted with ether and the residue recrystallized from 70 per cent alcohol. 0.15 gm. of sativic acid melting at 163–165°C. was obtained.

*Analysis.* 0.1371 gm. substance: $CO_2$ 0.3101, $H_2O$ 0.1306.
Calculated for $C_{18}H_{36}O_6$. C 62.1, H 10.3.
Found. C 61.7, H 10.6.

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[12] Lewkowitsch, J., Chemical technology and analysis of oils, fats and waxes, London, 6th edition, 1921, i, 575.

The isolation of sativic acid from the oxidation products indicates the presence of linolic acid ($C_{18}H_{32}O_2$) in this fraction.

The aqueous mother liquors from the tetrahydroxystearic and dihydroxystearic acids were examined for linusic acid ($C_{18}H_{36}O_8$) but only a small amount of acid melting at 135–140°C. was obtained. Linolenic acid is not present in this fraction.

*Examination of the Intermediate Fraction of Unsaturated Acids.*

The 30 gm. of acids whose barium salts were insoluble in the benzene-ether mixture and whose lead salts were ether-soluble, were again converted into their barium salts and extracted with the benzene-ether mixture. Only a small amount of the barium salts was now insoluble in this solvent.

The insoluble barium salt was decomposed with hydrochloric acid and the fatty acid extracted with ether. 0.9 gm. of acid was obtained having an iodine number of 83. 0.6 gm. of this acid was reduced with hydrogen and colloidal palladium by the method of Paal. 0.58 gm. of crystalline acids was obtained which after recrystallization from alcohol melted at 56–61°C. Mixed with palmitic acid, it melted at 52–62°C.

*Analysis.* 0.2147 gm. substance: $CO_2$ 0.5878, $H_2O$ 0.2380.
Calculated for $C_{16}H_{32}O_2$. C 75.0, H 12.5.
Found. C 74.7, H 12.4.

These analytical results indicate that the reduced acid is palmitic and that the unsaturated acid in the fat is a $C_{16}$ acid of the oleic series.

The barium salts soluble in the benzene-ether mixture, when decomposed with hydrochloric acid and extracted with ether, yielded 26.8 gm. of acid having an iodine number of 125. 16 gm. of this acid were dissolved in dry ether, cooled to 0°C., and saturated with bromine. 0.8 gm. of ether-insoluble bromides was obtained which blackened at 230°C.

*Analysis.* 0.1532 gm. substance: AgBr 0.2354.
Calculated for $C_{20}H_{32}O_2Br_8$. Br 67.8.
" " $C_{18}H_{30}O_2Br_6$. " 63.3.
Found. Br 65.4.

These results suggest a mixture of arachidonic octobromide and a hexabromide. There was not sufficient material to separate these by extraction with hot benzene.

0.5 gm. of an ether-soluble, petroleum ether-insoluble tetrabromide was obtained which after repeated extraction with petroleum ether melted and blackened at 135–140°C.

*Analysis.* 0.1891 gm. substance: AgBr 0.2436.
Calculated for $C_{18}H_{32}O_2Br_4$. Br 53.3.
" " $C_{18}H_{30}O_2Br_6$. " 63.3.
Found. Br 54.8.

The analysis indicates impure linolic tetrabromide.

25.7 gm. of liquid bromides soluble in petroleum ether were obtained. This dibromide fraction was debrominated by boiling with alcoholic hydrogen chloride and zinc dust. The resulting ethyl esters formed during this debromination were saponified and the acids obtained by decomposing the soap and extracting with ether. 14 gm. of acids having an iodine number of 107 were obtained. 3 gm. of this acid were reduced with hydrogen and colloidal palladium and the resulting solid acid crystallized twice from 95 per cent alcohol. 1.33 gm. of crystals melting at 62–65°C. were obtained. The filtrate yielded 0.3 gm. of acid melting at 61–62°C.

*Analyses.*
*Fraction 1.* 0.1518 gm. substance: $CO_2$ 0.4211, $H_2O$ 0.1732.
Found. C 75.7, H 12.8.
*Fraction 2.* 0.1498 gm. substance: $CO_2$ 0.4146, $H_2O$ 0.1715.
Found. C 75.5, H 12.8.
Calculated for $C_{18}H_{36}O_2$. C 76.0, H 12.7.
" " $C_{16}H_{32}O_2$. " 75.0, " 12.5.

These analytical results indicate a mixture of palmitic and stearic acids. The unsaturated acids of the dibromide fraction are, therefore, a mixture of oleic acid and a $C_{16}$ acid of the oleic series. The high iodine number (107) indicates the presence of a small amount of more unsaturated acids which did not separate as crystalline bromides during the bromination.

*Examination of the Saturated Acids.*

The saturated acids from the lead salt separations and those from crystallization of the crude acid mixtures were joined. These acids weighed 149 gm. After recrystallization from 95 per cent alcohol, 129 gm. of crystalline acids were obtained which melted at 53–55°C. When mixed with pure myristic acid (m.p. 54°C.)

the melting point was lowered, indicating that this was not myristic acid. An attempt was made to crystallize fractionally this mixture from alcohol but a satisfactory separation could not be obtained.

TABLE IV.

*Distillation of Methyl Esters of Saturated Acids.*

| Fraction. | Temperature. | Pressure. | Ester. | Acid from ester. | Acid after crystallization. | Melting point. |
|---|---|---|---|---|---|---|
| | °C. | mm. Hg | gm. | gm. | gm. | °C. |
| 1 | Up to 160 | 1.5–1.6 | 31.4 | 29.2 | 21.1 | 56–56.5 |
| 2 | 160–161 | 1.6–1.7 | 17.7 | 16.6 | 14.5 | 55–56.5 |
| 3 | 161–168 | 1.8 | 11.4 | 10.7 | 9.0 | 56–58 |
| 4 | 168–172 | 1.5–1.8 | 16.3 | 15.3 | 11.8 | 59–62 |
| 5 | 172–175 | 1.5–1.7 | 10.5 | 9.6 | 8.5 | 64–66 |

TABLE V.

*Analysis of Saturated Acids.*

| Fraction. | Neutralization value. | Analysis. | | | | | Calculated composition. | |
|---|---|---|---|---|---|---|---|---|
| | | Substance. | $CO_2$ | $H_2O$ | C | H | $C_{14}H_{28}O_2$ | $C_{16}H_{32}O_2$ |
| | | gm. | gm. | gm. | per cent | per cent | per cent | per cent |
| 1 | 214 | 0.1541 | 0.4232 | 0.1756 | 74.9 | 12.8 | 100 | |
| 2 | 211 | 0.1471 | 0.4052 | 0.1677 | 75.1 | 12.8 | 90 | 10 |
| 3 | 207 | 0.1519 | 0.4198 | 0.1723 | 75.4 | 12.7 | 60 | 40 |
| 4 | 203 | 0.1559 | 0.4324 | 0.1775 | 75.7 | 12.7 | 30 | 70 |
| 5 | 196 | 0.1553 | 0.4318 | 0.1769 | 75.9 | 12.7 | 10 | 90 |
| Calculated for | | | | | | | | |
| $C_{14}H_{28}O_2$ | 197 | | | | 76.0 | 12.8 | | |
| $C_{16}H_{32}O_2$ | 219 | | | | 75.0 | 12.5 | | |

These acids were converted into the methyl esters by refluxing for 4 hours with 200 cc. of methyl alcohol and 10 cc. of sulfuric acid. 131 gm. of methyl esters were obtained. 90 gm. of the esters were fractionally distilled at a pressure of 1.5 to 1.8 mm. of mercury. Each of the five fractions was saponified, the soap decomposed with dilute sulfuric acid, and the liberated fatty

acids extracted with ether. The ether solutions were washed free from sulfuric acid, dried over anhydrous sodium sulfate, and the ether removed. The resulting acids were weighed, crystallized once from alcohol, and the melting points determined. The results are given in Table IV.

The crystalline acids from the above distillation were analyzed for C and H and the neutralization values determined. From the analysis, the percentage of palmitic and stearic acids was calculated for each fraction. The results are tabulated in Table V.

These analytical results show that the saturated acids consist of a mixture of 68 per cent palmitic acid and 32 per cent stearic acid. The mother liquor from the crystallization of fraction 1 was examined for myristic acid. The alcoholic solution was concentrated and the residue crystallized into two fractions. The top fraction (2.9 gm.) melted at 55–56.5°C. and appeared to be identical with fraction 1. The bottom fraction (0.36 gm.) melted at 53–55°C., but when mixed with myristic acid, lowered its melting point.

*Analysis.* 0.1533 gm. substance: $CO_2$ 0.4192, $H_2O$ 0.1742.
Calculated for $C_{14}H_{32}O_2$. C 75.0, H 12.5.
" " $C_{14}H_{28}O_2$. " 73.7, " 12.3.
Found. C 74.6, H 12.7.

These results indicate impure palmitic acid. Myristic acid is not present in this fraction.

## SUMMARY.

700 gm. of the fat of the corpus luteum, freed from acetone- and alcohol-insoluble and cadmium chloride-precipitable phosphatides, yielded 524 gm. of fatty acids and 105 gm. of unsaponifiable material. From the unsaponifiable material, 58 gm. of cholesterol were crystallized. 52 per cent of the fatty acids are present as glycerides, the other 48 per cent existing as free fatty acids. 35 per cent of the total fatty acids are saturated and 65 per cent are unsaturated. The experimental work presented in this paper indicates the following composition of the fatty acid mixture: 25 per cent palmitic ($C_{16}H_{32}O_2$), 11.2 per cent stearic ($C_{18}H_{36}O_2$), 1.6 per cent ($C_{16}H_{30}O_2$), 32.8 per cent oleic ($C_{18}H_{34}O_2$), 16.6 per cent linolic ($C_{18}H_{32}O_2$), 8 per cent arachidonic ($C_{20}H_{32}O_2$), and 4.8 per cent of a new hexa unsaturated acid of the $C_{20}$ series having the formula $C_{20}H_{34}O_2$.

# THE CHEMICAL INVESTIGATIONS OF CORPUS LUTEUM.

## V. THE LIPOIDS OF THE ACETONE EXTRACT.

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The ultimate understanding of the physiology of the ovary will be based not only on the isolation and identification of the so called active principle but also upon the exact chemical information as to the other constituents of the gland. At various times there has been a tendency in the study of the ovary to assign to the phosphatide fraction the specific activity of the gland. Our investigation of the phosphatide from the acetone extract of corpus luteum tended to show that this was ordinary lecithin with a phosphorus to nitrogen ratio of 1 to 1. We expect that this lecithin will give on hydrolysis the usual fatty acids shown by Levene and others to be incorporated in the lecithin molecule.

While the phosphatide elaborated in the corpus luteum may not be active in a specific way yet it is undoubtedly a very important factor in metabolism. Fenger[1] showed that the ductless glands were very rich in phosphatides, the corpus luteum of pregnancy containing fifteen times as much phosphatide as the muscle fiber used as a standard. Corner[2] also showed that the phosphatide content of the corpus luteum of sows diminished as pregnancy developed. We expect to repeat our investigation of the lipoids of corpus luteum on material gathered at various known stages of pregnancy. Wesson[3] has shown that in the metabolism of arachidonic acid the arachidonic acid increase in fasting animals corresponds somewhat to the partial exhaustion of the glycogen stores in the animals and he suggests that the accumulation of

[1] Fenger, F., *J. Biol. Chem.*, 1916, xxvii, 303.
[2] Corner, G. W., *J. Biol. Chem.*, 1917, xxix, 141.
[3] Wesson, L. G., *J. Biol. Chem.*, 1925, lxv, 235.

arachidonic acid in the tissues may be caused by a slowing up under these conditions of an arachidonic acid utilization which is dependent upon the simultaneous oxidation of glucose. Another suggestion he makes is that the increase may be due to the increased demands on the fats for energy under these conditions. We are interested in considering the possibility that in the utilization of the fats by the body the unsaturated fatty acids pass through the phosphatide stage. By the study of the chemistry of the neutral fat menstruum of corpus luteum compared with the chemistry of the phosphatides with particular reference to the unsaturated fatty acid content of the arachidonic type found in both and the correlation of this if possible with the stages of the sexual cycle represented, we hope to gain some insight in regard to the relation and importance of these phosphatides to the metabolism of the ovary. Before we took up this work though, we thought it worth while to carry out this preliminary examination of the more easily procurable, pharmaceutically important corpus luteum.

## EXPERIMENTAL.

Our sample represented carefully collected, hand-dissected material, dried immediately after collection below 40°C. in the commercial way. It is representative of the material found on the market in that it has been taken from such a large number of cattle as to represent a good average sample. The material contained 4.5 per cent moisture and 5.4 per cent ash[4] and the dried material represented six parts of the fresh corpus luteum substance.

4.073 kilos of this were ground to pass through a No. 20 sieve. This material was thoroughly extracted four times with shaking in an atmosphere of nitrogen with 28 liters of pure acetone.

*Examination of the Acetone Extracts.*—These extracts were separately concentrated[5] to 2.5, 1.5, 0.5, and 0.5 liters, respectively, and allowed to stand for several weeks in the cold. Crystalline material separated from the first three acetone extracts, which was

[4] Fullerton, B., and Heyl, F. W., *J. Am. Pharm. Assn.*, 1924, xiii, 194.

[5] All distillations in this work were made under reduced pressure, at 40°C. or below in the presence of nitrogen. When solutions were allowed to stand they were saturated with nitrogen, stoppered, and allowed to remain in the ice box. Solids were dried in a black vacuum desiccator to prevent action of the light.

centrifuged off, washed with a little acetone, and dried. The precipitate from the fourth acetone extract was more gummy in nature than the others. This was crystallized from a small quantity of alcohol and the crystalline material added to that from the first three acetone extracts. The main filtrates and washings from the above described crystals were joined and evaporated to 1 liter and allowed to stand for several weeks in the cold. Another crop of crystalline material was obtained which was joined with the main fraction. Weight of the combined crystalline fraction, 14.50 gm. A similar fraction, of 3.99 gm., separating from the acetone extract from 1 kilo of corpus luteum has been shown by us[6] to consist of cholesteryl palmitate, potassium stearate, and fatty acids. As the previous work on this fraction had indicated the possibility of other substances being present besides those reported, this material was submitted to a systematic re-examination.

*Examination of the Crystalline Material Separating on Concentration of the Acetone Extracts of Corpus Luteum.*—This crystalline material probably contained phosphatides as was shown by the following analysis:

*Analysis.* 0.1710, 0.1963 gm. substance: cc. N/20 NaOH, 26.32, 29.31. P 0.85, 0.83.

This material was submitted to a fractional crystallization from hot alcohol. 2.798 gm. of this was insoluble in hot alcohol. This was examined separately and the results of this examination, together with the results[7] from a similar insoluble fraction, from our previous study of the acetone extraction of the 1000 gm. of corpus luteum, showed this material to consist of a mixture of the amino acids leucine, isoleucine, and possibly valine. The hot alcoholic solution deposited on cooling typical cholesteryl palmitate crystals. On treating these crystals with ether, 0.01 gm. was insoluble. This insoluble material was not a soap and melted sharply at 84–85°C. This was suggestive of the presence in this fraction of some cholesteryl esters of some of the fatty acids higher in the series than palmitic. There was not sufficient material though for confirmation of this point. The ether was

[6] Hart, M. C., and Heyl, F. W., *J. Am. Pharm. Assn.*, 1924, xiii, 17.
[7] Hart, M. C., and Heyl, F. W., *J. Am. Pharm. Assn.*, 1925, xiv, 770.

removed from the ether-soluble part of this fraction and the residue was entirely soluble in benzene. The ether- and benzene-soluble material was crystallized several times from alcohol. 4.1 gm. of very uniform microscopic flat, plate-like crystals of cholesteryl palmitate melting at 69–71°C. were obtained.

Two other crystalline fractions were obtained by concentrating the alcoholic filtrates from the cholesteryl palmitate crystallization. These on systematic examination yielded 0.064 gm. more of ether-soluble cholesteryl palmitate melting at 69–71°C. and traces of ether-insoluble fractions that decomposed at 253°C. and 243°C. respectively. These ether-insoluble fractions were similar in character to the alcohol-insoluble part of this fraction which had a decomposition point of 257–258°C. and which has been shown to consist of a mixture of amino acids.

By further concentrations of the filtrate from the above crystalline fractions another crop of crystals was obtained. On treatment with benzene a fraction, 0.04 gm. of material, decomposing at 268°C., was obtained. This was mostly soluble in water, yielding more of these amino acids on evaporation which decomposed at 254°. The benzene-soluble part of this fraction when crystallized from alcohol yielded a small amount of crystals melting at 75–77°C. This was emulsified with water and extracted with ether and the residue from the ether extract crystallized from alcohol. About 0.05 gm. of crystals melting at 77–78°C. was obtained. This was not a soap and again indicated the possibility of other cholesteryl esters besides cholesteryl palmitate being present in the acetone extract of corpus luteum.

The filtrate from these above described crystals contained phosphatides and was joined with the alcoholic filtrate from the crystallization of the gummy precipitate separating from the fourth acetone extraction. This alcoholic solution was concentrated to a volume of 50 cc. This solution showed the presence of 0.37 gm. of phosphorus (equivalent to about 15 gm. of lecithin) when analyzed by the Neumann method.

*Analysis.* 2.00 cc. of sample: cc. N/5 NaOH, 66.80.
P 0.72.

The alcohol was removed from this solution and the residue crystallized from 100 cc. of absolute alcohol, the solution being

cooled overnight in the salt-ice mixture. A vaseline-like solid separated which was centrifuged off. This vaseline-like material was entirely soluble in ether and a systematic crystallization from acetone and alcohol yielded nothing of a definite nature. The alcohol was removed from the alcohol-soluble part of this fraction, and the residue taken up in 20 cc. of ether. This ether solution which deposited no insoluble material on standing for 24 hours at 0°C. was diluted to 200 cc. with cold absolute alcohol, and centrifuged from a slight amount of insoluble material. This clear alcoholic solution was precipitated with 200 cc. of a saturated alcoholic cadmium chloride solution. A light yellow flocculent precipitate was formed. This was centrifuged off, washed thrice with alcohol, and dried to constant weight *in vacuo*. It weighed 9.416 gm.

This cadmium chloride salt was partially purified by moistening with 95 per cent alcohol and digesting in the cold with 100 cc. of ether containing 2 per cent alcohol. This process of extraction was repeated twice using two 50 cc. portions of the 2 per cent alcoholic ether solution. The insoluble material was then centrifuged off and dried to constant weight *in vacuo*. Weight 7.064 gm. This was analyzed for nitrogen and phosphorus.

*Analyses.* 0.1038, 0.1005 gm. substance: cc. N/50 $NH_3$, 5.54, 5.43.
N 1.50, 1.51.
0.0715, 0.0714 gm. substance: cc. N/50 NaOH, 33.6, 33.48.
P 2.60, 2.60.
N:P::1.28:1.

These results indicate that this material is of the nature of an impure lecithin. Lack of material prevented further purification and study of this fraction.

The systematic examination of the filtrate from this cadmium chloride salt yielded nothing of interest.

The main acetone filtrates and washings from the above described crystalline precipitates were concentrated to a syrup and dissolved again in 1 liter of pure acetone. On standing in the cold for several days a precipitate separated out. This was centrifuged off, washed thrice with acetone, and dried *in vacuo*.

*Examination of Material Separating from the Acetone Extract of Corpus Luteum on Resolution in Acetone.*—This was a brown

fatty appearing material. Weight 9.025 gm. The phosphorus determination indicated that this material might be of the nature of an impure phosphatide.

*Analysis.* 0.1967, 0.1824 gm. substance: cc. N/20 NaOH, 52.43, 48.80. P 1.48, 1.48.

This material was crystallized from 75 cc. of hot alcohol. The crystals obtained were thoroughly emulsified with water to remove any soap present, and extracted with ether. The ether extract was evaporated to dryness and crystallized from 175 cc. of alcohol. 1.012 gm. of crystals melting at 64–68°C. were obtained. These gave on two more recrystallizations from alcohol, 0.616 gm. of cholesteryl palmitate melting at 69–71°C.

The soap solution from which this cholesteryl palmitate had been extracted with ether was filtered and evaporated to dryness. The small amount of residue was crystallized from 10 cc. of alcohol, taken up in water, and precipitated with hydrochloric acid. The precipitated acid was extracted with ether, the ether removed, and the residue crystallized from 3 cc. of alcohol, the alcohol solution being cooled in the salt-ice mixture. A small amount of the fatty acid was filtered off and dried on a clay plate. This melted at 65°C. This was evidently impure stearic acid from a potassium soap we have shown[6] to be present in corpus luteum.

The main alcoholic filtrate from the crystallization of the cholesteryl palmitate was evaporated to dryness. A brown semi-crystalline solid was obtained. This was taken up in 25 cc. of warm ether and the ether solution cooled in the salt-ice mixture. A gelatinous precipitate formed which was filtered off and washed with ether. This material was crystallized again from 25 cc. of ether, cooled, filtered, washed as before, and dried to constant weight *in vacuo*. 0.792 gm. of a white powder was obtained. This turned to clear oil at 182–183°C. and ran down the melting point tube at 185°C.

Fränkel and Fonda[8] have shown the presence of a phosphosulfatide melting at 180–200°C. and separating from the ether extract of corpus luteum. Fränkel and Gilbert[9] have also de-

[8] Fränkel, S., and Fonda, M., *Biochem. Z.*, 1923, cxli, 379.
[9] Fränkel, S., and Gilbert, O., *Biochem. Z.*, 1921, cxxiv, 206.

scribed a phosphosulfatide (Hirnsaure) isolated from the ether-insoluble material from the hot alcoholic extract of brain tissue. 0.3655 gm. of our material, however, when analyzed for sulfur gave negative results. Cerebrosides were not present, as was shown by the negative orcinol test.

This substance was analyzed for phosphorus and nitrogen. These results expressed in terms of atomic ratios showed the presence of 2.2 atoms of nitrogen to 1 of phosphorus.

*Analyses.* 0.0963, 0.0977 gm. substance: cc. N/50 $NH_3$, 8.2, 8.6.
0.0634 gm. substance: cc. N/20 NaOH, 28.57.
Found. N 2.39, 2.47, P 2.49.

These results suggest the presence of sphingomyelin. Lack of material, however, prevented confirmation of this point.

The ether filtrates from the separation of the above described phosphatide were concentrated to about 20 cc., held at 0°C. for several hours, and filtered from some insoluble material. The ether was removed and the residue taken up again in 20 cc. of fresh ether. This ether solution separated no more insoluble material on standing in the cold, and it was diluted to 125 cc. with cold absolute alcohol. A little gelatinous material separated on standing in the cold. This was centrifuged off and the clear alcoholic filtrate precipitated with 125 cc. of saturated alcoholic cadmium chloride solution. A light yellow flocculent cadmium chloride salt was obtained. This was centrifuged off, washed thrice with alcohol, and dried to constant weight *in vacuo*. 1.390 gm. of this cadmium chloride compound were obtained.

This cadmium chloride salt was partially purified by moistening with 95 per cent alcohol and digesting in the cold with 15 cc. of ether containing 2 per cent alcohol. This process of extraction was repeated twice, using two 7 cc. portions of the 2 per cent alcoholic ether solution. The insoluble material was then centrifuged off and dried to constant weight *in vacuo*. Weight 0.899 gm. This was analyzed for nitrogen and phosphorus.

*Analyses.* 0.1038, 0.1023 gm. substance: cc. N/50 $NH_3$, 5.33, 5.28.
N 1.44, 1.45.
0.0683, 0.0694 gm. substance: cc. N/20 NaOH, 33.20, 33.84.
P 2.69, 2.70.
N:P::1.19:1.

These results indicate an impure lecithin. Lack of material prevented further work on the confirmation of this point.

The alcoholic filtrate from the above described cadmium chloride salt contained no more cadmium chloride-precipitable material. This was taken to dryness, taken up in ether, filtered from excess insoluble cadmium chloride, and the ether solution evaporated to dryness. By crystallization from alcohol this residue yielded 0.032 gm. of fatty acids melting at 50–54°C. This was not further examined.

The main acetone filtrates from which the above described material weighing 9.025 gm. was obtained separated nothing more on standing in the cold. The acetone was removed and the oily residue taken up with 2 liters of absolute alcohol. On standing in the cold this solution gradually separated some white material. This was centrifuged off, washed thrice with absolute alcohol, and dried to constant weight *in vacuo*. This material appeared fatty in nature and weighed 5.66 gm.

*Examination of Material Separating from the Alcoholic Solution of the Acetone Extract of Corpus Luteum.*—This material contained a small amount of phosphorus.

*Analysis.* 0.1104, 0.1194 gm. substance: cc. N/20 NaOH, 5.27, 5.33. P 0.26, 0.25.

This material was crystallized three times from 50 cc. of ether by taking up in warm ether and then cooling the ether solution in the salt-ice mixture. The final crystals obtained were then crystallized from 100 cc. of hot alcohol. This material gave the Liebermann-Burchard test for cholesterol. It softened at 55°C. and melted at 65°C. This material appeared to be a cholesteryl ester contaminated with some impurity difficult to separate by crystallization. Fränkel and Fonda[8] separated glycerol tripalmitate from the ether extract of corpus luteum, and it was thought that this contaminating impurity might be of that nature. The crystals were dissolved in ether and an extraction by means of 10 per cent sodium hydroxide yielded no alkali-soluble material in this fraction, proving the absence of free fatty acids. The ether solution was washed thoroughly with water, dried, and the ether removed. The residue from the evaporation of the ether was crystallized from 30 cc. of warm ether, the ether solution being

cooled in the salt-ice mixture. The crystalline precipitate was filtered off and dried to constant weight *in vacuo*. It weighed 2.1931 gm.

This crystalline material was hydrolyzed by boiling under a reflux with 200 cc. of a 6 per cent alcoholic potassium hydroxide solution for 12 hours. The alcohol was then evaporated off, the residue taken up in water, and extracted with ether. This ether extract was washed and dried and on evaporation yielded 0.8910 gm. of typical cholesterol-like crystals. These gave the ordinary color tests for cholesterol and when recrystallized from 40 cc. of 95 per cent alcohol gave 0.430 gm. of flat plate-like crystals melting at 143–145°C. A mixed melting point with pure cholesterol was not depressed.

The soap solution from which the cholesterol had been extracted with ether was precipitated by hydrochloric acid in the cold, the solution boiled, cooled, and filtered. This process of digesting with hydrochloric acid was repeated. 1.3192 gm. of fatty acids were obtained which on one crystallization from 40 cc. of alcohol melted at 56–57°C. This corresponds to the melting point of a 50 per cent mixture of palmitic and stearic acids.

Calculating the cholesterol over to cholesteryl palmitate, the 0.8999 gm. of cholesterol found is equivalent to 1.438 gm. of cholesteryl palmitate. The other part of this fraction of 2.1931 gm. can be explained on the assumption that it consisted of the fat tristearin.

The ether filtrates from the above described mixtures yielded on systematic examinations crystals of impure cholesteryl palmitate melting at 66–69°C. Nothing further of interest was demonstrated in this filtrate.

The main filtrates and washings from the above described fatty material were concentrated and held in the cold at various concentrations. Nothing more of a crystalline nature separated. This extract was made up to 1 liter and a total solid determination showed the presence of 647.6 gm. (15.90 per cent).

To this acetone, alcohol-soluble fraction of the acetone extract of corpus luteum was added a similar fraction prepared in an identical manner from 1.6 kilos of the corpus luteum. The entire alcohol solution measuring 2 liters was precipitated with 4.2 liters of a saturated alcoholic solution of cadmium chloride. A

white flocculent precipitate formed. This was centrifuged off, washed thrice with acetone, twice with absolute alcohol, and dried *in vacuo*. Weight 153.20 gm. The filtrates and washings from the above precipitate on concentration and treatment with more alcoholic cadmium chloride solution yielded 18.90 gm. more of this substance.

*Purification of Cadmium Chloride Compound from the Acetone Extract of Corpus Luteum.*—This was a slightly yellow powder. It was attempted to purify 10 gm. of this material by the toluene-ether process described by Levene[10] but the results were not satisfactory. Also Levene's ether crystallization method when tried on another 10 gm. sample did not give results warranting its use on the main fraction.

152 gm. of the cadmium chloride salt were decomposed by the method used by Levene and Simms.[10] The salt was dissolved in 475 cc. of warm chloroform and poured into 600 cc. of methyl alcohol containing 30 gm. of ammonia. After a short time the solution was centrifuged. The precipitate of cadmium hydroxide was washed once with a mixture of 40 cc. of chloroform and 50 cc. of alcoholic ammonia. The filtrates and washings were concentrated to a vaseline-like consistency. This was thoroughly emulsified with 550 cc. of water, a little salt added, and the lecithin precipitated with 550 cc. of ice-cold acetone. This mixture was allowed to stand 12 hours in a salt-ice mixture and then filtered off. This process of emulsification and precipitation was repeated twice more. The lecithin was then taken up in 500 cc. of ether, cooled for 5 hours in the salt-ice mixture, filtered, and evaporated to dryness. The residue was taken up in 125 cc. of ether and the ether solution added to 1.5 liters of cold 95 per cent alcohol. The solution was turbid with the separation of some vaseline-like material. This solution was cooled for 12 hours in the salt-ice mixture, filtered, and precipitated with 1100 cc. of saturated alcoholic cadmium chloride. The mixture was cooled thorougly and the precipitated cadmium chloride compound separated by means of the centrifuge. The precipitate was washed twice with 350 cc. of 95 per cent alcohol and once with 300 cc. of absolute alcohol. It was dried to constant weight *in vacuo*. Weight 77.56 gm. This was ampouled.

[10] Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1921, xlviii, 185.

The acetone aqueous filtrates from the emulsification of this cadmium chloride salt were joined together, evaporated to a syrup. The syrup contained crystals of ammonium chloride which were separated by treatment with absolute alcohol. The results of the examination of the alcoholic filtrate from this ammonium chloride will be reported on later.

The cadmium chloride salt obtained after emulsification was analyzed.

*Analyses.* 0.1033, 0.1051, 0.1060 gm. substance: cc. N/50 $NH_3$, 5.21, 5.02, 4.98.
N 1.41, 1.34, 1.32.
0.2021, 0.2020 gm. substance: $BaSO_4$, 0.0005, 0.0003.
S 0.03, 0.02.
0.0714, 0.0714 gm. substance: cc. N/20 NaOH, 33.55, 34.12.
P 2.60, 2.64.
N:P::1.15:1.

To determine the amount of cephalin contamination, 2.0650 gm. were hydrolyzed by boiling for 12 hours with 100 cc. of 10 per cent hydrochloric acid. The mixture was cooled and filtered from the fat. The fat was digested twice with 20 cc. portions of 5 per cent hydrochloric acid, cooled, and filtered. The filtrates were taken down on the steam bath to a small volume, nearly neutralized with 25 per cent sodium hydroxide, filtered again, and the filtrate again concentrated to about 7 cc. It was then made just alkaline with sodium hydroxide, then acid with acetic acid, and then filtered into a 20 cc. volumetric flask and made up to the mark.

2 cc. samples of this were analyzed for amino nitrogen by the micro Van Slyke and for total nitrogen by the micro Kjeldahl method.

*Analysis.* 2.00, 2.00 cc. samples: cc. N at 25°C. and 746 mm. pressure, 0.61, 0.59, average, 0.60.
Blank: 0.11, 0.14, 0.11, 0.11 cc., average 0.12 cc.
N in the amino form, 0.000266 gm.
*Analysis.* 2.00, 2.00 cc. samples: cc. N/50 $NH_3$, 10.08, 9.94.
Total N 0.002824, 0.002785 gm., average, 0.002805 gm.
$\frac{0.000266}{0.002805}$ = 9.48 per cent of the total nitrogen in the amino form.

These analytical results indicate the presence of appreciable

quantities of cephalin as well as other nitrogen-containing impurities in this fraction.

*Further Purification of This Lecithin Cadmium Chloride Compound.*—10 gm. of the cadmium chloride compound were moistened with 3 cc. of 95 per cent alcohol, 110 cc. of ether containing 2 per cent alcohol added, and the mixture allowed to stand for 12 hours. It was centrifuged and the insoluble material washed again with 50 cc. of ether containing the 2 per cent alcohol. The insoluble material was then dissolved in the hot in 100 cc. of a mixture of 2 parts ethyl acetate and 1 part 80 per cent alcohol, filtered, and allowed to crystallize. Microscopic lumps of needle-like crystals radiating out from a common center were formed. The material was entirely homogeneous. These crystals were centrifuged off, washed with ethyl acetate and ether, and recrystallized again from 75 cc. of the ethyl acetate-alcohol mixture. These crystals after being centrifuged off, washed with ethyl acetate and ether, and dried to constant weight *in vacuo*, weighed 7.02 gm.

*Analyses.* 0.0618, 0.0624 gm. substance: cc. N/20 NaOH, 33.03, 33.83. P 2.96, 3.00.
0.1038, 0.1050 gm. substance: cc. N/50 $NH_3$, 5.00, 5.30. N 1.35, 1.41.
N:P::1.026:1.00.

1.6123 gm. were hydrolyzed by boiling for 8 hours with 100 cc. of 10 per cent hydrochloric acid. The filtrates from the fat cake were concentrated and made up to a volume of 20 cc. in the usual manner.

*Analyses.* 2.00, 2.00 cc. samples: cc. N/50 $NH_3$, 7.43, 7.46.
Total N 0.0020818, 0.0020902 gm., average, 0.002086 gm.
Blanks: cc. N at 24.5°C. and 757 mm. pressure; 0.16, 0.18, 0.17, average 0.17.
2.00, 2.00 cc. samples: cc. N at 24.5°C. and 757 mm. pressure; 0.19, 0.20, 0.21, average 0.20. Average $NH_3$ N from 2.00 cc., 0.03 cc., 0.0000166 gm.

$$\frac{0.0000166}{0.002086} = 0.8 \text{ per cent of the total nitrogen in the amino form.}$$

These analytical results indicate that we have here a pure lecithin cadmium chloride salt, free from any appreciable contamination with cephalin. The results of the hydrolysis of this lecithin will be reported in a separate paper.

The alcoholic filtrate from the precipitation of the lecithin cadmium chloride salt was concentrated to dryness, taken up in

ether, and filtered from the insoluble excess cadmium chloride present. This ether solution contained the fat of corpus luteum. The results[11] of the chemical study of this fat are reported in a separate paper.

SUMMARY.

The results of the chemical investigation of the acetone extract of corpus luteum show that there are present cholesterol, cholesteryl palmitate, potassium stearate, leucine, isoleucine, valine, possibly sphingomyelin, tristearin, a lecithin which was prepared pure with a nitrogen and phosphorus ratio of 1 to 1, and free from contaminating cephalin impurities and fat, the chemistry of which we have reported on in the preceding paper.

[11] Cartland, G. F., and Hart, M. C., *J. Biol. Chem.*, 1925, lxvi, 619.

# THE FORMATION OF LACTIC ACID BY DEPANCREATIZED DOGS.

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Our interest in the ability of diabetic individuals to form lactic acid had its origin in our (Briggs, Koechig, Doisy and Weber, 1923–24) finding that after the administration of large doses of insulin to fasted dogs there was, among other changes, an increase in the lactic acid concentration of the blood. Reasoning from this, it seemed possible that the diabetic defect might lie in the organism's inability to perform the transformation carbohydrate → lactic acid. A perusal of the literature revealed two investigations having a direct bearing on this point. (1) Sass (1914) found that strychnine convulsions produced a smaller decrease of titratable alkalinity of the blood of depancreatized than of normal dogs and concluded that depancreatized dogs are less able to form lactic acid. (2) Embden and Isaac (1917) found that by perfusion of the livers of depancreatized dogs with defibrinated blood containing lactic acid and glucose the lactic acid decreased and that glucose increased by approximately the same amount, whereas with the livers of normal dogs they obtained an increase in lactic acid and a decrease in glucose.

The results of the investigations just referred to formed the basis upon which we ventured the hypothesis that insulin affects carbohydrate metabolism by increasing the rate of formation of lactic acid. Upon examining the evidence supporting this hypothesis, we noticed that Sass' conclusions were based on three experiments and that lactic acid determinations were not made. To determine the reliability of this evidence, we have studied the capacity of depancreatized dogs to form lactic acid. Since our

work was begun, Loebel, Barr, Tolstoi, and Himwich (1924)[1] have published their studies in which phlorhizinized dogs were used, but because of the uncertain relation of phlorhizin to pancreatic diabetes, and because our work brings out some additional points, we desire to record our evidence on the formation of lactic acid by diabetic animals.

In view of the recent development of the energetics of muscular contraction by Hill and Meyerhof,[2] our work has had an added interest. Most of the data underlying the deductions of Hill and of Meyerhof have been obtained on frog muscle. Whether the glycogen $\rightleftarrows$ lactic acid reaction could be carried over to mammalian muscle was not clear at the beginning of our experiments 2½ years ago. More recent work (Hill (1924), Meyerhof and Himwich (1924)) indicates that many of the processes of frog and mammalian muscle are similar. Such being the case, it is not surprising that Woodyatt (1913) found low lactic acid maxima for the muscles of his phlorhizinized dogs in which he showed that the muscle glycogen was very low.

Though the dependence of the lactic acid formation upon glycogen during the anaerobic phase and the nature of the substance oxidized in the restitution phase to convert the lactic acid back to glycogen seems well established for normal isolated frog muscle, there seems to be insufficient evidence that only carbohydrate (or lactic acid) can be utilized in the restitution phase. The painstaking investigation of Krogh and Lindhard (1920) seems to show that their subjects on high fat diets must have burned fat in performing work. If, as Hill and Meyerhof seem to think, glycogen $\rightarrow$ lactic acid is a prerequisite for contraction then possibly in the subjects of Krogh and Lindhard the change in lactic acid $\rightarrow$ glycogen was effected by the combustion of fat, although the work on the fat diet required the expenditure of about 10 per cent more energy. Lusk (1925; Anderson and Lusk, 1917) seems to be in accord with this view.

[1] This paper discusses the topic of lactic acid formation by diabetics so well that a repetition in our paper is unnecessary. The reader is referred to the paper of Loebel *et al.*, for an interesting résumé of some of the relations of lactic acid in metabolism.

[2] Results of the many published investigations are summarized in Hill's lectures (1924) and Meyerhof, O., Chemical dynamics of life phenomena, Philadelphia, 1924.

However, the evidence on this point is not entirely clear. *A priori*, from our conception of complete diabetes and numerous failures to obtain clear-cut evidence of the transformation of fat into carbohydrate, we should expect that if depancreatized dogs form lactic acid from glycogen during the anaerobic phase, they would burn fat or protein during the aerobic restitution phase. In Hill's (1924) laboratory it has been found that the formation of lactic acid during short periods of exercise and removal during recovery in a diabetic (severity not stated) appear to be the same as in normal individuals. Furusawa (1925) working in Hill's laboratory, found that the respiratory quotient of the excess metabolism of prolonged exercise upon a high fat diet approached 1 (0.92 to 0.97). These investigations have seemingly led Hill (1924) to believe that carbohydrate is chiefly concerned in the oxidation in the restitution phase, and that when carbohydrate is lacking, fat is transformed before it can be utilized. It is somewhat difficult to harmonize this with the conception that a complete diabetic cannot burn sugar, yet is capable of movements.

Cathcart (1925) in his examination of the literature seems inclined to the view that work increases protein catabolism and Meyerhof, Lohmann, and Meier (1925) find a formation of $NH_3$ in surviving mammalian muscle. Although Winfield (1914–15) did not detect an alteration of the amount or character of the fatty acids of frog muscle after prolonged stimulation, and Parnas (1921) concluded that the metabolism of the surviving muscle of depancreatized and normal frogs is identical, we have felt that an attempt to examine the possibility of substances other than carbohydrates being oxidized in the restitution phase would be worth while. We hope to present our data on this point soon.

## EXPERIMENTAL.

Male dogs were depancreatized under ether anesthesia using the common aseptic precautions. Females were avoided to obviate any complications due to pregnancy (Carlson and Drennan, 1911). Following the operation, the dog was injected twice daily with insulin and fed liberally on lean beef. After a week or 10 days, the incision was generally healed and then the animal's insulin was withdrawn.

For the benefit of those who, like ourselves, are not experienced

operators, we may say that depancreatization is a relatively simple procedure. The pancreas is loosened from its connection with the duodenum by scraping it loose with a piece of gauze and the finger tips (see Hédon, 1891). A few pieces of connective tissue holding minute quantities of pancreas, which are not loosened easily, are picked off with blunt forceps. We find it advisable to preserve as much of the blood supply to the duodenum as possible, and have rarely ligated more than two or three blood vessels. It is, we have since been informed, ordinary technique with surgeons to cover raw surfaces with peritoneum or omentum to prevent adhesions. Not knowing this at the start of our work, we had a high mortality; post mortem, most of the animals showed adhesions. We tried fastening a fold of the omentum about the raw surface of the duodenum with two sutures to hold it in place. Upon autopsy, a week or more later, it was generally found that the omentum had grown fast and that there were no adhesions. Since instituting this procedure, it has been very rare that we have lost an animal prematurely.

The treatment of our animals subsequent to the withdrawal of insulin has varied considerably, some being fed, others fasted, and still others treated with adrenalin and fasted. With the exception of the last group, the urines were separated into 24 hour quantities by catheterization. When the D:N had fallen to the characteristic value (generally in 3 to 5 days), the effect of exercise upon blood lactic acid was studied. In the strychnine experiments, we used 0.2 to 0.3 mg. strychnine nitrate per kilo. With the exception of the two dogs treated with adrenalin, which required much larger doses, generalized convulsions came on in about 30 minutes. Besides the blood taken before the injection of strychnine, other samples were drawn prior to convulsions during the period of muscular contraction induced by stimulation; the last sample was generally taken after the dog had had a spontaneous generalized convulsion. Frequently it was necessary to resort to artificial respiration and ether to save our animals from the respiratory failure brought on by the strychnine.

Attention should be called to the enormous doses of strychnine required to produce convulsions in adrenalin-treated depancreatized dogs. The protocols of Loebel, Barr, Tolstoi, and Himwich indicate that they likewise resorted to large doses in their

adrenalin-treated phlorhizinized dogs. Whether this is a vasomotor effect of the adrenalin or due to an extreme muscular weakness associated with the low glycogen content which adrenalin produces in diabetic dogs (Table II), we will leave to the pharmacologist.

Two dogs were treated with adrenalin to remove the sugar rapidly after the withdrawal of insulin. After a few days, they were given strychnine nitrate and lactic acid determinations made on the blood. Since these experiments were performed Cori (1925) has published his investigations showing the increase of lactic acid in the blood of rabbits and cats following the injection of adrenalin. This finding can hardly vitiate our experiments, as we gave the adrenalin at least 3 hours prior to the strychnine and in one case found an increase in lactic acid of over 100 per cent 30 minutes after the injection of strychnine, and over 400 per cent 15 minutes later.

To eliminate any extraneous drug actions which might be confusing, we have performed experiments on two dogs in which the muscular movements were produced by leading the dogs at a run up and down the laboratory. In these experiments, Dogs 14 and 15, there is an unmistakable production of lactic acid. Electrical stimulation of the hind leg of Dog 14 also produced lactic acid.

Samples of muscle and liver were taken from a few of our dogs for analysis. Analysis of glycogen, lower carbohydrates, and lactic acid were conducted by methods very similar to those used by Cori and Cori (1925). Clausen's (1922) method was used for lactic acid determinations. Urinary analyses were promptly made on fresh 24 hour urines, care being taken to avoid fermentation of the sugar. Glucose was determined by the method of Folin and Peck (1919) and total nitrogen by the usual Kjeldahl procedure.

## DISCUSSION.

Contrary to the findings of Sass, which have been rather widely quoted, we find that strychnine convulsions in depancreatized dogs produce a large increase in blood lactic acid. This agrees with the result of Loebel, Barr, Tolstoi, and Himwich on phlorhizinized dogs and disposes of the idea that diabetics are unable to form lactic

acid. It seems that other recent work (Tolstoi, 1924, and Cajori and Crouter, 1924) indicates that glycolysis in diabetic blood is not less rapid than in normal blood as had been found in several laboratories. It seems probable then that diabetics may form lactic acid very readily.[3]

Returning to our discussion of the data given in this paper, we find less regularity of D:N than we expected. In general, the dogs fed on lean beef had higher D:N values than the fasted animals and their figures were usually between 2 and 3, but a little lower than the classical ratio. In spite of the traces of pancreas remaining in Dogs 2 and 3, the former had a D:N of 2.73 the 9th day, and the latter 2.76 on the 7th day after the withdrawal of insulin. This indicates that the remnant of pancreas was exerting little or no effect on the carbohydrate metabolism.

The ratios of the fasting dogs (Nos. 4, 5, 6, and 14) are rather interesting. No. 5 had a D:N above 2 for 11 days and only on the 12th and 13th days fell below 2. The other animals' ratios fell more rapidly, presumably because of their poorer nutritive condition at the beginning of fasting. At the start of the fast, Dog 14 weighed less than 70 per cent of his weight at operation and showed but one D:N above 2 after the withdrawal of the insulin. These animals were fasted to make an extreme test of the ability of depancreatized animals to form lactic acid.

Glancing at Table I, it may be seen that depancreatized dogs produce lactic acid during strychnine convulsions and running. This indicates the validity of the Hill-Meyerhof theory of the anaerobic phase of contraction even in the diabetic state. In Dog 15, the blood lactic acid is quite high after a few minutes of exercise and over half an hour after the termination of the exercise the value is still much above the control. We have not studied

[3] In an earlier paper we reported increases in blood lactic acid following the injection of normal fasted dogs with insulin. A number of investigators (Tolstoi, Loebel, Levine, and Richardson, 1923–24, Best and Ridout, 1925, Servantie, 1925, and Cori, 1925) have failed to confirm our findings. We have repeated our work using added precautions and in a series of three fasted and three well fed dogs find increases of lactic acid varying from 71 to 270 per cent. Two fed and one depancreatized dogs failed to show an increase, and in view of the failure of others to confirm our results it seems that the appearance of increased amounts of lactic acid is not an invariable result of the injection of insulin.

TABLE I.

*Blood Lactic Acid during Muscular Movements of Strychnine Convulsions and Running.*

| Dog No. | Strychnine nitrate. | Blood lactic acid. | | Remarks. |
|---|---|---|---|---|
| | | Control. | During muscular movements. | |
| | *mg. per kg.* | *mg. per 100 cc.* | *mg. per 100 cc.* | |
| 1 | 0.34 | 17 | 19 92 107 117 | Normal dog. |
| | 0.25 | 26 | 87.5 178 213 | Depancreatized but receiving insulin; died from overdose of strychnine. |
| 2 | 0.21 | 31 | 51.5 42 | Insulin given between drawing of second and third blood samples. |
| | 0.27 | 29.5 | 63 89.5 123 89.5 | Ether given to save dog. |
| | 0.27 | 37.5 | 71 97.5 | |
| 3 | 0.30 | 21 | 40.5 46.5 64.5 | Normal prior to pancreas removal. |
| | 0.20 | 38.5 | 50.5 56 109 | Ether given to save dog after drawing last blood. |
| | 0.21 | 35.5 | 81 113 128 | Died during convulsion after taking last blood. |
| 4 | 0.20 | 42 | 60 83 89 122 | |
| | 0.18 | 42 | 63 83 | |
| 5 | 0.19 | 42 | 63 94 | |
| | 0.20 | 40 | 48 113 117 | No generalized convulsion but greatly increased tonus. |
| 6 | 0.50 | 28 | 67 | Dog was very weak and convulsions were mild. Strychnine given in two injections. Last blood taken from heart ½ min. after heart stopped. |
| 8 | 1.10 | 16 | 39 89 | Treated with adrenalin; see protocol. |

TABLE I—*Concluded.*

| Dog No. | Strychnine nitrate. | Blood lactic acid | | | Remarks. |
|---|---|---|---|---|---|
| | | Control. | During muscular movements. | | |
| | *mg. per kg.* | *mg. per 100 cc.* | *mg. per 100 cc.* | | |
| 9 | 0.65 | 34 | 36 | 36 | Dog had one mild general convulsion ½ min. after taking third blood and heart stopped; muscles limp and flaccid. Treated with adrenalin. |
| 14 | | 29 | 35 | 38 | Led at a dog trot 9½ min. between first and second samples. |
| | | 18 | 35 | 25 | Stimulated leg electrically for 15 min. |
| 15 | | 20 | 34 | | Ran 5 min. |
| | | 24 | 96 | 41 | Second sample after 9½ min. of running, third 36 min. later. |
| | | 20 | 58 | 33 | Second sample after 14 min. of exercise; third sample 30 min. later. |

For more complete information about the above animals reference may be made to the protocols at the end of the paper. Animals 2, 3, and 15 were fed on lean beef continuously throughout the experiments; Nos. 4, 5, 6, and 14 were fasted; Nos. 8 and 9 were fasted and in addition received adrenalin to reduce their store of carbohydrate.

normal dogs and consequently do not know whether or not the diabetic can dispose of this increased lactic acid as promptly as the normal.

Apparently Dog 9 did not produce lactic acid. This animal had been treated with adrenalin and was exceptionally weak. He did not respond to the first injection of strychnine (0.2 mg. per kilo) so other injections were made. 2 minutes after taking the third blood, the dog suddenly had a mild tremor and his heart stopped. His failure to respond to strychnine might have been due to his

very low muscle carbohydrates; the total lactic acid produced in a bicarbonate solution containing $CHCl_3$ was only 0.15 per cent (see Meyerhof and Himwich. 1924). To be sure, the muscle of Dog 8 produced even less lactic acid in $NaHCO_3$ and $CHCl_3$ but this animal had severe strychnine convulsions just prior to death which may have depleted the lactic acid precursors. In both of these

TABLE II.

*Carbohydrates and Lactic Acid in Muscle and Liver of Depancreatized Dog.*

| Dog No. | Lower carbohydrates. | Glycogen. | Initial lactic acid. | Total of Columns 2, 3, 4. | Lactic acid formed. | Liver glycogen. | Days depancreatized. | Days without insulin. | Remarks. |
|---|---|---|---|---|---|---|---|---|---|
| | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | | | |
| | | | | | 0.449 | | | | Normal dog. |
| 4 | | | | | 0.442 | | 24 | 8 | Muscle taken after death due to strychnine. |
| 5 | | | | | 0.436 | | 29 | 13 | Dog died 2 days after last experiment. |
| 6 | | | | | 0.172 | | 14 | 5 | Muscle taken after death due to strychnine; 12 hrs. in $CHCl_3$ water. |
| 8 | 0.037 | 0.013 | 0.061 | 0.111 | 0.125 | 0.09 | 22 | 5 | Muscle taken after death due to strychnine. Animal had been treated with adrenalin. Lactic acid formed in $NaHCO_3$ solution and $CHCl_3$. |
| 9 | 0.01 | 0.02 | 0.13 | 0.16 | 0.15 | 0.08 | 19 | 3 | Death due to strychnine. Animal had been treated with adrenalin. Lactic acid formed in $NaHCO_3$ solution and $CHCl_3$. |
| 14 | | | | | | 0.21 | 42 | 7 | Killed by shooting. |
| 15 | | | | | | 0.69 | 32 | 13 | " " " |

dogs' muscles there is a fair agreement between lactic acid formed on standing and the sum of lactic acid, glycogen, and lower carbohydrate in the fresh muscle. In two of the dogs which had undergone a similar preparatory treatment, Woodyatt (1913) was unable to detect the presence of glycogen, yet the muscles formed 0.107 and 0.167 per cent of lactic acid. That this lactic acid prob-

ably came in part from the lower carbohydrate is indicated by our data in Table II.

Although the values given below may be too small due to the method used, the lactic acid formed by muscle in $CHCl_3$ water in Dogs 4 and 5 is indicative of a high muscle glycogen. Muscle of Dog 5 which was fasted 14 days possessed almost as much lactic acid (0.436 per cent) precursor as we found in a normal dog (0.449 per cent). No. 6 which had such low D:N values formed much less lactic acid in $CHCl_3$ water even though he died from strychnine before much lactic acid had diffused into the blood.

The values for liver glycogen (Table II) in the adrenalin-treated depancreatized dogs correspond well with values already reported (Cruickshank, 1913–14, Fisher and Lackey, 1925) for diabetic animals. Nos. 14 and 15 received glucose by mouth shortly before death and had 0.21 and 0.69 per cent liver glycogen. It is possible that these data indicate the ability to form and store glycogen temporarily in the liver, but they are too few to warrant serious consideration.

## CONCLUSIONS.

1. Depancreatized dogs retain the capacity of forming lactic acid during strychnine convulsions or running.

2. Depancreatized dogs exhibit a remarkable tenacity for the lactic acid precursor substances of muscle; treatment with adrenalin reduces these precursors rapidly to low values.

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## PROTOCOLS.

Our laboratory notes on three exercise experiments which are typical of the others are given in the protocols.

Analyses were not made on urines from Dog 1. Postmortem: gross; a small quantity of pancreatic tissue which was later verified histologically.

*Dog 2.*

Operated Mar. 21; weight 7.54 kilos. No insulin after Apr. 11; fed lean beef.

| Date. | Weight. | Food eaten. | Urinary glucose, 24 hrs. | Urinary nitrogen, 24 hrs. | D:N | Remarks |
|---|---|---|---|---|---|---|
| *Apr.* | *kg.* | *gm.* | *gm.* | *gm.* | | |
| 12 | 6.15 | 710 | 49.7 | 7.94 | 6.25 | |
| 13 | 6.02 | 600 | 41.8 | 15.2 | 2.75 | Part of urine lost. |
| 14 | 5.72 | 150 | 26.7 | 9.77 | 2.72 | |
| 15 | 5.58 | 350 | 33.8 | 11.94 | 2.83 | |
| 16 | 5.40 | 250 | 20.0 | 10.37 | 1.92 | Experiment in afternoon. Urine includes period of experiment. Ether given to save dog. |
| 17 | 4.82 | 340 | | | 2.75 | Volume not recorded. Lost night urine. |
| 18 | 5.03 | 400 | 15.84 | 6.4 | 2.48 | Experiment in morning. Ether given to save dog. Urine includes period of experiment. |
| 19 | 4.62 | 300 | 15.4 | 8.14 | 1.89 | |
| 20 | 4.56 | 300 | 14.6 | 5.35 | 2.73 | |

Postmortem: Histological examination revealed small nodule of pancreatic tissue in duodenal wall.

*Dog 3.*

Operated Apr. 11; weight 9.8 kilos. No insulin after Apr. 22; fed cooked lean beef.

| Date. | Weight. | Food eaten. | Urinary glucose, 24 hrs. | Urinary nitrogen, 24 hrs. | D:N | Remarks. |
|---|---|---|---|---|---|---|
| *Apr.* | *kg.* | *gm.* | *gm.* | *gm.* | | |
| 23 | 8.85 | 700 | 68.5 | 16.85 | 4.06 | |
| 24 | 8.50 | 470 | 42.0 | 13.16 | 3.19 | |
| 25 | 8.30 | 590 | 52.8 | 15.94 | 3.30 | |
| 26 | 8.15 | 510 | 47.7 | 15.72 | 3.03 | |
| 27 | 7.95 | 220 | 39.9 | 13.74 | 2.90 | |
| 28 | 7.62 | 450 | 48.6 | 14.68 | 3.30 | Experiment in afternoon. Ether was given to save dog. |
| 29 | 7.50 | 480 | 30.6 | 11.07 | 2.76 | |
| 30 | | | | | | Experiment. Dog died from overdose of strychnine. |

Postmortem: Minute trace of pancreas in muscle wall of duodenum in region of duct.

*Dog 4.*

Operated Apr. 25; weight 8.0 kilos. No insulin after May 11; last food on May 11.

| Date. | Weight. | Food eaten. | Urinary glucose, 24 hrs. | Urinary nitrogen, 24 hrs. | D:N | Remarks. |
|---|---|---|---|---|---|---|
| *May* | *kg.* | *gm.* | *gm.* | *gm.* | | |
| 12 | 6.70 | 0.00 | 18.93 | 3.18 | 5.95 | |
| 13 | 6.40 | | 13.51 | 3.65 | 3.70 | |
| 14 | 6.25 | | 9.76 | 3.82 | 2.55 | |
| 15 | 6.08 | | 10.98 | 4.75 | 2.31 | |
| 16 | 5.87 | | 11.21 | 4.70 | 2.39 | |
| 17 | 5.75 | | 5.26 | 3.58 | 1.47 | Experiment 1, ether given to save dog at conclusion of experiment. |
| 18 | 5.62 | | 10.18 | 5.12 | 1.99 | |
| 19 | 5.43 | | | | | Experiment 2, no urine analysis made. Was unable to catheterize and obtain urine. Dog died. |

Postmortem: Gross, no pancreas; microscopic, no acinous nor islet tissue.

*Strychnine Experiment 1.*

Weight 5.75 kilos.

10.20 a.m. Blood 1. Arterial-femoral artery by skin puncture.

10.23 a.m. 1.15 cc. strychnine (0.2 mg. per kilo).

10.30 a.m. Contralateral reflexes on tapping knee. This stimulation was continued at the rate of 40 per min.

10.33 a.m. Spontaneous reflexes.

10.49 a.m. Blood 2. Taken while dog was in a mild spasm.

10.57 a.m. Blood 3. Taken after a series of mild spasm elicited by tapping table.

11.00 a.m. Blood 4. Rather strong spontaneous contraction.

11.05 a.m. Blood 5. Strong violent contractions, artificial respiration by pumping chest with hands.

11.08 a.m. Blood 6. Passed into a severe tetanic convulsion. Artificial respiration was applied as before.

11.10 a.m. Ether was given to save the dog.

*Dog 5.*

Operated May 6; weight 7.5 kilos. No insulin after May 21; no food after May 21.

| Date. | Weight. | Food eaten. | Urinary glucose, 24 hrs. | Urinary nitrogen, 24 hrs. | D:N | Remarks. |
|---|---|---|---|---|---|---|
| *May* | *kg.* | *gm.* | *gm.* | *gm.* | | |
| 22 | 6.93 | 0.00 | | | | |
| 23 | 6.65 | | 20.25 | 2.43 | 8.29 | |
| 24 | 6.38 | | 15.82 | 4.00 | 3.95 | |
| 25 | 6.32 | | 13.91 | 4.96 | 2.80 | |
| 26 | 6.18 | | 14.58 | 5.19 | 2.80 | |
| 27 | 5.97 | | 13.55 | 5.50 | 2.45 | |
| 28 | 5.82 | | 11.86 | 5.22 | 2.27 | |
| 29 | 5.65 | | 18.70 | 5.79 | 4.07 | Experiment. Ether given at conclusion of experiment to save dog. |
| 30 | 5.45 | | 9.83 | 4.79 | 2.08 | |
| 31 | 5.28 | | 11.51 | 5.52 | 2.08 | |
| *June* | | | | | | |
| 1 | 5.12 | | 12.34 | 5.50 | 2.24 | |
| 2 | 4.92 | | 12.60 | 5.35 | 2.35 | Experiment. Ether given to save dog. |
| 3 | 4.81 | | | | 1.53 | Volume not recorded, part of urine lost. |
| 4 | 4.69 | | | | 1.79 | Killed. No volume recorded. Could not obtain quantitative collection. Urine obtained from bladder after dog was killed. |

Postmortem: Gross, no pancreas; microscopic, no acinous nor islet tissue.

*Dog 6.*

Operated Aug. 19; weight 5.27 kilos. No insulin after Aug. 27; no food after Aug. 27.

| Date. | Weight. | Food eaten. | Urinary glucose, 24 hrs. | Urinary nitrogen, 24 hrs. | D:N | Remarks. |
|---|---|---|---|---|---|---|
| *Aug.* | *kg.* | *gm.* | *gm.* | *gm.* | | |
| 27 | 4.25 | 0.00 | | | 0.093 | |
| 28 | 4.11 | | 14.91 | 4.30 | 3.47 | |
| 29 | 4.01 | | 7.84 | 4.24 | 1.85 | |
| 30 | 3.90 | | | | (1.49) | Unable to catheterize, and urine |
| 31 | 3.74 | | | | (1.49) | voided on Aug. 31. Two samples obtained. |
| *Sept.* | | | | | | |
| 1 | 3.50 | | 6.41 | 4.60 | 1.39 | |
| 2 | 3.36 | | | | 1.72 | A 3½ hrs. urine sample, just before experiment. |

Postmortem: No pancreas present.

*Dog 8.*

Operated Aug. 25; weight 6.04 kilos. No insulin after Sept. 11; no food after Sept. 11.

| Date. | Weight. | Food eaten. | Urinary glucose, 24 hrs. | Urinary nitrogen, 24 hrs. | D:N | Remarks. |
|---|---|---|---|---|---|---|
| *Sept.* | *kg.* | *gm.* | *gm.* | *gm.* | | |
| 12 | 4.80 | 0.00 | | | | 11.15 a.m. 0.2 cc. adrenalin 1:1000. |
| | | | | | | 3.30 p.m. 0.2 " " 1:1000. |
| 13 | 4.62 | | | | 8.62 | This only a specimen collected during day. |
| 14 | 4.50 | | 6.14 | 3.74 | 1.64 | |
| 15 | 4.43 | | 6.41 | 3.86 | 1.66 | |
| 16a* | 4.40 | | | | 0.70 | 8.30 a.m. 0.25 cc. adrenalin 1:1000. |
| 16b | | | | | 4.14 | 12.15 p.m. 0.50 " " 1:1000. Collected urine at 12.05 p.m. |
| 16c | | | | | 4.97 | 3.45 p.m. strychnine experiment. Collected urine at 3.30 p.m. Experiment. |

* Night urine grossly contaminated; discarded. Urine obtained by catheter was analyzed and contained 3.58 mg. N per cc. and 2.5 mg. sugar per cc.

Postmortem: Gross, no pancreatic tissue, microscopic, no acinous nor islet tissue.

*Strychnine Experiment.*

Dog lies very quiet in cage. No movement.

3.39 p.m. Blood 1, arterial.

3.45 p.m. 1.0 mg. strychnine subcutaneously.

3.51 p.m. No response on stimulation. 0.90 mg. strychnine subcutaneously.

3.57 p.m. No response. 1.0 mg. strychnine subcutaneously.

4.00 p.m. No response. 1.0 mg. strychnine intraperitoneally.

4.06 p.m. No response. 1.0 mg. strychnine intraperitoneally.

4.07 p.m. Single twitches on stimulation.

4.12 p.m. General spasm which consisted chiefly of a stretching of legs and passing into a condition of rigor.

4.15 p.m. Blood 2, arterial; seems well oxygenated.

4.16 p.m. Dog becomes limp, irritability lost; respiration ceased and was continued by artificial means until dog died.

4.30 p.m. Dog died.

4.31 p.m. Blood 3 taken from heart.

*Dog 9.*

Operated Aug. 23; weight 10.65 kilos. No insulin after Sept. 8; no food after Sept. 8.

| Date. | Weight. | D:N | Remarks. |
|---|---|---|---|
| *Sept.* | *kg.* | | |
| 9 | 7.76 | 3.50 | Urine voided during day. |
| 10 | 7.16 | 3.08 | " " " night. |
| | | | 0.25 cc. adrenalin at 9.00 a.m. |
| 10a | | 3.45 | Collected at 12.00 m. |
| | | | 0.25 cc. adrenalin at 12.15 p.m. |
| 10b | | 3.63 | Collected at 2.40 p.m. |
| | | | 0.25 cc. adrenalin at 3.30 p.m. |
| | | | 0.25 " " " 5.30 " |
| 11 | 6.56 | 2.71 | Collected at 8.00 a.m. |
| | | | 0.25 cc. adrenalin at 8.00 a.m. |
| | | | Experiment at 11.25 a.m. |
| | | | Dog died from strychnine effects. |

Postmortem: Gross, no pancreatic tissue; microscopic, no pancreatic tissue.

*Strychnine Experiment.*

Sept. 11. Weight 6.43 kilos, after giving adrenalin. Dog in bad shape. Lies absolutely quiet in cage. Moribund condition.

11.25 a.m. Blood 1, arterial.

11.30 a.m. 1.25 mg. strychnine subcutaneously.

11.42 a.m. No signs of increased irritability. 0.50 mg. strychnine subcutaneously.

11.45 a.m. No response on stimulation. 1.0 mg. strychnine subcutaneously.

11.50 a.m. No response on stimulation. 0.50 mg. strychnine intraperitoneally. Stimulated by tapping knee, 40 per min. for 5 min.; single twitch only response elicited.

11.55 a.m. Blood 2, arterial.

11.57 a.m. 1.0 mg. strychnine intraperitoneally.

12.01 p.m. Stimulated 50 per min. for 4 min.

12.05 p.m. Blood 3, dark, taken during a mild general convulsion which consisted of a sudden stretching of the dog's leg, from which he passed into a perfectly limp condition within 30 seconds with all irritability lost. Respiration ceased at 12.06½ and heart stopped at 12.07.

*Dog 14.*

Operated Apr. 30; weight 8.06 kilos. No insulin after June 3; given ground, cooked lean, washed meat.

| Date. | Weight. | Food eaten. | Urinary glucose, 24 hrs. | Urinary nitrogen, 24 hrs. | D:N | Remarks. |
|---|---|---|---|---|---|---|
| *June* | *kg.* | *gm.* | *gm.* | *gm.* | | |
| 5 | 5.60 | 300 | | | 1.66 | Quantitative collection begun from this date. |
| 6 | 5.60 | 300 | 26.4 | 9.15 | 2.89 | |
| 7 | 5.35 | 150 | 12.4 | 6.53 | 1.90 | |
| 8 | 5.25 | 70 | 5.85 | 4.51 | 1.29 | |
| 9 | 5.10 | 0.00 | 3.91 | 3.00 | 1.30 | Food discontinued; dog refuses to eat. Experiment 1. |
| 10 | 4.95 | 0.00 | 3.19 | 2.48 | 1.29 | Experiment 2. |
| 11 | 4.770 | | | | | Dog killed. |

Postmortem: Gross, no pancreas; microscopic, no acinous or islet tissue.

*Dog 15.*

Operated May 7; weight 7.66 kilos. No insulin after May 25; washed, ground, cooked lean beef given throughout.

| Date. | Weight. | Food eaten. | Urinary glucose, 24 hrs. | Urinary nitrogen, 24 hrs. | D:N | Remarks. |
|---|---|---|---|---|---|---|
| *May* | *kg.* | *gm.* | *gm.* | *gm.* | | |
| 26 | 6.25 | 300 | | | 0.965 | Volume not measured. |
| 27 | 5.85 | 300 | 33.8 | 10.4 | 3.25 | |
| 28 | 5.82 | 300 | 32.4 | 11.8 | 2.75 | |
| 29 | 5.80 | 300 | 33.8 | 12.2 | 2.76 | |
| 30 | 5.65 | 300 | 34.6 | 13.7 | 2.52 | |
| 31 | 5.52 | 300 | 29.0 | 11.05 | 2.63 | |
| *June* | | | | | | |
| 1 | 5.35 | 300 | 24.8 | 10.75 | 2.30 | Experiment 1. |
| 2 | 5.15 | 300 | 24.4 | 10.25 | 2.38 | |
| 3 | 5.05 | 300 | 25.0 | 10.70 | 2.33 | Experiment 2. |
| 4 | 4.90 | 300 | 20.8 | 9.20 | 2.26 | |
| 5 | 4.65 | 300 | 20.2 | 9.95 | 2.03 | Experiment 3. |
| 6 | 4.46 | 300 | 18.6 | 9.77 | 1.90 | |
| 7 | 4.42 | 300 | 12.85 | 8.40 | 1.53 | |
| 8 | 4.43 | | | | | Dog killed by shooting. |

Postmortem: Gross, no pancreas; histological, no pancreas.

# BENZOYLATED AMINO ACIDS IN THE ANIMAL ORGANISM.

## I. THE BEHAVIOR OF HIPPURIC ACID FOLLOWING ITS ORAL ADMINISTRATION.*

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Benzoylated amino acids have frequently been used in metabolism studies, usually for the purpose of determining whether these compounds were oxidized in the animal body. Magnus-Levy (1) demonstrated that such substances were not oxidized but were eliminated unchanged in the urine after parenteral administration. On the other hand enteral administration has resulted in partial oxidation in some cases. Epstein and Bookman (2) found hippuric acid in the urine of rabbits after the administration of benzoyl leucine (method of administration not stated) and benzoyl alanine (*per os*). The procedure used in isolating and determining hippuric acid in their experiments was not reported. For this reason it was impossible to ascertain from their data whether any unaltered benzoyl leucine or benzoyl alanine was present in the urine. Lewis, Updegraff, and McGinty (3) noted that there was some oxidation of cystine after feeding rabbits dibenzoyl cystine. No oxidation occurred after the subcutaneous injection of this compound. They decided that some of the benzoyl groups were split off in the alimentary canal with subsequent oxidation of the liberated cystine. Csonka (4) concluded that his experiments did not support the theory that ingested hippuric acid was split before absorption since the excretion of benzoyl glycuronic

* A preliminary report of this work was presented before the American Society of Biological Chemists at St. Louis, December, 1923. (Griffith, W. H., *J. Biol. Chem.*, 1924, lix, p. li.)

acid was increased after the administration of benzoate but not after the administration of hippurate.

In experiments carried out for another purpose it was observed that the elimination of hippuric acid in rabbits after hippurate feeding was markedly increased by the simultaneous administration of glycine with the hippurate but was not affected by the administration of alanine. If hippuric acid were absorbed from the intestine and excreted by the kidneys without undergoing any change, this increased elimination after the feeding of hippurate and glycine would be difficult to explain. However, if hippuric acid were hydrolyzed to benzoic acid in the alimentary canal, then the administration of glycine with hippurate might result in an increased synthesis and output of hippuric acid just as after the feeding of benzoate and glycine (5). A study was made, therefore, of the behavior of hippuric acid after its oral administration in order to determine whether or not this compound was hydrolyzed to benzoic acid before absorption.

## EXPERIMENTAL.

In all of the experiments normal rabbits were used. As in the earlier experiments, the rate of excretion of free and combined benzoic acid was determined for the 6 hour period following the administration of the experimental substance. Free benzoic acid was determined according to the method of Raiziss and Dubin (6) and total benzoic acid according to the procedure of Kingsbury and Swanson (7). In the tables the figures for combined benzoic acid represent the difference between the free and total benzoic acid of the urine. These values represent extra combined benzoic acid, a correction having been made for the normal excretion of hippuric acid. For rabbits on a milk diet this amounts to 0.080 to 0.100 gm. per day.

### *1. The Rate of Excretion of Hippuric Acid after Subcutaneous, Intraperitoneal, and Oral Administration.*

It was previously shown that sodium hippurate (equivalent to 1 gm. of benzoic acid per kilo) was quantitatively eliminated in the 6 hour period after its intravenous injection (5). Table I shows the rate of excretion of hippuric acid after its subcutaneous

TABLE I.

*Excretion of Hippuric Acid (Calculated as Combined Benzoic) in the 6 Hour Period Following the Intraperitoneal and Subcutaneous Administration of Sodium Hippurate.*

| Rabbit No. | Weight. | Benzoic acid per kilo administered as sodium hippurate. | Extra combined benzoic acid excreted in 6 hr. period. | | Method of administration. |
|---|---|---|---|---|---|
| | *kg.* | *gm.* | *gm.* | *per cent of intake* | |
| 4 | 1.08 | 1.38 | 1.315 | 88 | Intraperitoneal. |
| 2 | 1.14 | 1.31 | 1.470 | 98 | " |
| 3 | 1.2 | 1.25 | 1.371 | 92 | " |
| 1 | 1.3 | 1.14 | 1.145 | 76 | " |
| 2 | 1.14 | 1.31 | 1.355 | 90 | Subcutaneous. |
| 3 | 1.2 | 1.25 | 1.439 | 96 | " |
| 1 | 1.3 | 1.14 | 1.441 | 96 | " |
| 11 | 2.27 | 0.67 | 1.142 | 75 | " |

TABLE II.

*Excretion of Hippuric Acid (Calculated as Combined Benzoic) in the 6 Hour Period Following the Oral Administration of Sodium Hippurate.*

| Rabbit No. | Weight. | Benzoic acid per kilo administered as sodium hippurate. | Extra combined benzoic acid excreted in 6 hr. period. | |
|---|---|---|---|---|
| | *kg.* | *gm.* | *gm.* | *per cent of intake* |
| 2 | 1.14 | 1.31 | 1.050 | 70 |
| 3 | 1.20 | 1.25 | 0.682 | 46 |
| 1 | 1.30 | 1.14 | 0.785 | 52 |
| 35 | 1.30 | 1.11 | 0.847 | 59 |
| 40 | 2.10 | 1.10 | 1.200 | 52 |
| 27 | 1.70 | 1.06 | 0.972 | 54 |
| | | 1.06 | 0.805 | 45 |
| 28 | 1.90 | 1.04 | 1.213 | 61 |
| 23 | 1.50 | 0.96 | 0.684 | 47 |
| | | 0.96 | 0.775 | 54 |
| 31 | 1.80 | 0.90 | 1.076 | 66 |
| | | 0.90 | 0.695 | 43 |
| 32 | 2.20 | 0.82 | 0.933 | 52 |
| 60 | 2.60 | 0.77 | 1.013 | 51 |
| 58 | 2.70 | 0.74 | 0.968 | 48 |
| 11 | 2.30 | 0.66 | 0.599 | 39 |
| Average of 16 experiments | | | | 52 |

and intraperitoneal administration. It is evident that there was very rapid absorption from the subcutaneous tissues and from the peritoneal cavity as well as rapid elimination through the kidneys. However, after the oral administration of sodium hippurate, the elimination of hippuric acid in the 6 hour period was much less than the elimination after parenteral administration. In sixteen experiments on thirteen rabbits (Table II) the average excretion in the 6 hour period was only 52 per cent of the ingested hippurate. Since there was no delay in the elimination of hippuric acid after its intravenous, subcutaneous, or intraperitoneal injection the delay after oral administration must have been due either to its hydrolysis to benzoic acid or to its slow absorption from the alimentary tract.

*2. The Rate of Excretion of Hippuric Acid after the Administration of Hippurate with Glycine, Alanine, or Hydrolyzed Proteins.*

If hydrolysis to benzoic acid occurred in the alimentary canal after the feeding of hippurate, then the rate of elimination of hippuric acid in the urine would depend upon the rate of synthesis of hippuric acid just as after the administration of sodium benzoate. Griffith and Lewis (5) found that 41 per cent of the ingested benzoate was eliminated in the urine as hippuric acid in the 6 hour period after the feeding of benzoate alone (equivalent to 1 gm. of benzoic acid per kilo) and that this value was increased to 71 per cent after the simultaneous feeding of benzoate and glycine. *The excretion of hippuric acid was similarly increased by administering glycine with sodium hippurate.* In thirteen experiments on eight rabbits (Table III) the average output of hippuric acid in the 6 hour period after the oral administration of hippurate and glycine was 79 per cent. This represented more than a 50 per cent increase over the average output of hippuric acid after feeding hippurate alone. In order to determine whether this increase was characteristic of amino acids in general, alanine was administered with hippurate. In nine experiments on six rabbits (Table III) the average excretion in the 6 hour period was only 57.6 per cent. The same results were obtained when the amino acids were injected subcutaneously.

Since glycine was so much more effective in increasing the elimination of hippuric acid than alanine, it was of interest to determine

the effect of feeding hippurate and a mixture of amino acids obtained by the enzymatic hydrolysis of proteins. Hydrolyzed gelatin, elastin, casein, edestin, glutenin, and egg albumen were used in these experiments (Table IV). The feeding of hippurate with gelatin or elastin, proteins which have a high content of glycine, resulted in a greater output of hippuric acid in the 6 hour period than the feeding of hippurate with casein, edestin, glutenin, or egg albumen, proteins which contain little or no glycine. In view of the fact that these results were practically identical with those obtained by feeding benzoate with glycine, alanine, or hydrolyzed proteins (5, 8), it was concluded that hippuric acid was hydrolyzed to benzoic acid in the digestive tract. The increased rate of excretion of hippuric acid when glycine was administered with hippurate was considered to represent an increased rate of synthesis of hippuric acid from benzoic acid (formed by the hydrolysis of hippurate in the intestine) and an excess of preformed glycine.

*3. The Absorption of Sodium Hippurate and of Sodium Benzoate from the Small Intestine.*

The experiments reported above do not exclude the possibility that some hippurate was absorbed from the intestine without undergoing hydrolysis. However, the following experiments, in which the rates of absorption of benzoate and hippurate were compared by direct determinations of the extent of absorption of these substances from intestinal loops, demonstrated that hippuric acid was not readily absorbed from the small intestine of the rabbit. In these loop experiments, rabbits were anesthetized with urethane or ether and either one or two sections of small intestine, 12 to 15 inches in length, were ligated and cannulated at each end. Care was taken to interfere as little as possible with the blood supply of the sections of intestine used. After washing out the loop with warm saline solution a known amount of either benzoate or hippurate was introduced into the loop. At the end of the absorption period, usually 30 to 45 minutes, the contents of the loop were thoroughly washed out. This solution was boiled to coagulate protein, filtered, and analyzed for unabsorbed benzoic or hippuric acid. In nine rabbits there was an average absorption of 31 per cent of the benzoate (Table

TABLE III.

*The Excretion of Hippuric Acid in the 6 Hour Period Following the Oral Administration of Sodium Hippurate Alone and of Sodium Hippurate with Glycine or Alanine. (Glycine and Alanine Administered Either Orally or Subcutaneously.)*

| Rabbit No. | Hippuric acid excreted in the 6 hr. period after administration of: | | | | | | Glycine. | Alanine. | Hippuric acid fed. |
|---|---|---|---|---|---|---|---|---|---|
| | Sodium hippurate alone. | | Sodium hippurate and glycine. | | Sodium hippurate and alanine. | | | | |
| | *gm.* | *per cent of intake* | *gm.* | *per cent of intake* | *gm.* | *per cent of intake* | *mol* | *mol* | *gm.* |
| 23* | 1.020 | 47 | 1.960 | 93 | 1.422 | 67 | 3 Subcutaneously. | 3 Subcutaneously. | 2.11 |
| | 1.137 | 54 | 1.806 | 85 | 1.471 | 70 | 3 *Per os.* | 3 *Per os.* | 2.11 |
| 27 | 1.180 | 45 | 2.155 | 80 | 1.557 | 59 | 2 *Per os.* | 3 *Per os.* | 2.64 |
| | 1.426 | 54 | 2.263 | 86 | 1.638 | 62 | 2 Subcutaneously. | 3 Subcutaneously. | 2.64 |
| 31 | 1.580 | 66 | 2.210 | 93 | 1.290 | 54 | 2 Subcutaneously. | 3 *Per os.* | 2.38 |
| | 1.020 | 43 | 1.790 | 75 | | | 2 *Per os.* | | 2.38 |
| 32 | 1.368 | 52 | 2.150 | 81 | | | 2 *Per os.* | | 2.64 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 35 | 1.242 | 59 | 1.505 | 71 | | | 2.5 *Per os.* | | 2.11 |
| 40 | 1.760 | 52 | 2.460 | 72 | 1.270 | 38 | 2 *Per os.* | 3 *Per os.* | 3.37 |
| | | | 2.225 | 66 | 1.935 | 57 | 2 Subcutaneously. | 0.56 *Per os.* | 3.37 |
| | | | 2.535 | 75 | | | 0.56 *Per os.* | | 3.37 |
| 55 | 1.420 | 48 | 2.440 | 83 | 1.436 | 49 | 1.5 *Per os.* | 1.5 *Per os.* | 2.93 |
| 60 | 1.489 | 51 | 2.077 | 71 | 1.825 | 62 | 0.8 *Per os.* | 0.8 *Per os.* | 2.93 |
| Average..... | | 52 | | 79 | | 57.6 | | | |

* The weights of the rabbits used in this experiment are given in Table II.

TABLE IV.

*The Excretion of Hippuric Acid in the 6 Hour Period Following the Oral Administration of Sodium Hippurate Alone and of Sodium Hippurate with Hydrolyzed Proteins.*

| Date. | Hippuric acid. | | | Notes. |
|---|---|---|---|---|
| | Excreted. | | Ingested. | |
| **Rabbit 40. Weight 2.2 kilos.*** | | | | |
| *1923* | *gm.* | *per cent of intake* | *gm.* | |
| Jan. 4 | 1.760 | 52 | 3.374 | Control. |
| " 12 | 2.436 | 72 | 3.374 | Glycine (2 equivalents). |
| Feb. 15 | 1.284 | 38 | 3.374 | Alanine (2 " ). |
| " 23 | 2.278 | 68 | 3.374 | 5.0 gm. gelatin. |
| Mar. 2 | 1.562 | 46 | 3.374 | 5.0 " casein. |
| " 5 | 2.295 | 68 | 3.374 | 4.0 " " and glycine (0.56 equivalent). |
| " 7 | 2.523 | 75 | 3.374 | Glycine (0.56 equivalent). |
| " 9 | 1.560 | 46 | 3.374 | Control. |
| " 20 | 1.992 | 59 | 3.374 | Alanine (0.56 equivalent). |
| July 10 | 1.440 | 43 | 3.374 | Control. |
| " 11 | 1.667 | 49 | 3.374 | 3.3 gm. edestin. |
| " 12 | 2.400 | 71 | 3.374 | 1.65 " " and glycine (0.9 equivalent). |
| **Rabbit 60. Weight 2.6 kilos.*** | | | | |
| July 6 | 1.488 | 51 | 2.935 | Control. |
| " 7 | 2.078 | 71 | 2.935 | Glycine (0.8 equivalent). |
| " 8 | 1.828 | 62 | 2.935 | Alanine (0.8 " ). |
| " 10 | 1.520 | 52 | 2.935 | 3.3 gm. edestin. |
| " 11 | 2.410 | 82 | 2.935 | 1.65 " " and glycine (1.2 equivalents). |
| " 18 | 2.018 | 69 | 2.935 | 3.3 gm. gelatin. |
| **Rabbit 58. Weight 2.6 kilos.*** | | | | |
| July 2 | 1.420 | 48 | 2.935 | Control. |
| " 3 | 2.440 | 83 | 2.935 | Glycine (1.6 equivalents). |
| " 5 | 1.440 | 49 | 2.935 | Alanine (1.6 " ). |
| " 6 | 1.722 | 59 | 2.935 | 3.3 gm. glutenin. |
| " 7 | 2.050 | 70 | 2.935 | 3.3 " elastin. |
| " 8 | 1.769 | 60 | 2.935 | 3.3 " egg albumen. |

* The weights of these rabbits remained constant throughout the experiments.

TABLE V.

*The Absorption of Sodium Hippurate and of Sodium Benzoate from the Small Intestine.*

| Rabbit. | Loop. | Sodium hippurate calculated as benzoic acid. | | | Sodium benzoate calculated as benzoic acid. | | | Duration of experiment. |
|---|---|---|---|---|---|---|---|---|
| | | Administered. | Recovered. | Absorption. | Administered. | Recovered. | Absorption. | |
| | | *gm.* | *gm.* | *per cent* | *gm.* | *gm.* | *per cent* | *min.* |
| A | Single. | 1.365 | 1.268 | 7 | | | | 15 |
| | | | | | 1.365 | 0.970 | 29 | 15 |
| B* | Upper. | | | | 0.545 | 0.458 | 16 | 30 |
| | Lower. | 0.545 | 0.505 | 7 | | | | 30 |
| | Upper. | 0.545 | 0.532 | 2.5 | | | | 30 |
| | Lower. | | | | 0.545 | 0.368 | 32 | 30 |
| C | Upper. | | | | 0.545 | 0.303 | 44 | 60 |
| | Lower. | 0.545 | 0.530 | 3 | | | | 60 |
| D | Upper. | 0.545 | 0.486 | 11 | | | | 30 |
| | Lower. | | | | 0.545 | 0.425 | 22 | 30 |
| E* | Upper. | 0.545 | 0.532 | 2.5 | | | | 30 |
| | Lower. | | | | 0.545 | 0.346 | 36.5 | 30 |
| | Upper. | | | | 0.545 | 0.372 | 32 | 30 |
| | Lower. | 0.545 | 0.515 | 5.5 | | | | 30 |
| F | Upper. | 0.545 | 0.497 | 9 | | | | 30 |
| | Lower. | | | | 0.545 | 0.401 | 26.5 | 30 |
| G | Upper. | 0.545 | 0.505 | 7 | | | | 60 |
| | Lower. | 0.545 | 0.505 | 7 | | | | 60 |
| H | Single. | 0.545 | 0.472 | 13 | | | | 45 |
| K | Upper. | 0.545 | 0.509 | 6.5 | | | | 45 |
| | Lower. | | | | 0.545 | 0.422 | 22.5 | 45 |
| M | Single. | 1.090 | 1.030 | 5.5 | 1.090 | 0.952 | 13 | 45 |
| N | Single. | 0.545 | 0.505 | 7 | | | | 45 |

TABLE V—*Concluded.*

| Rabbit. | Loop. | Sodium hippurate calculated as benzoic acid. | | | Sodium benzoate calculated as benzoic acid. | | | Duration of experiment. |
|---|---|---|---|---|---|---|---|---|
| | | Administered. | Recovered. | Absorption. | Administered. | Recovered. | Absorption. | |
| | | *gm.* | *gm.* | *per cent.* | *gm.* | *gm.* | *per cent* | *min.* |
| O* | Upper. | 0.545 | 0.432 | 21 | | | | 45 |
| | Lower. | | | | 0.545 | 0.268 | 51 | 45 |
| | Upper. | 0.545 | 0.456 | 16 | | | | 45 |
| | Lower. | | | | 0.545 | 0.280 | 49 | 45 |
| P† | Single. | 1.500 | 1.245 | 17 | | | | 390 |
| Average of all experiments..... | | | | 8.7 | | | 31 | |

* In the experiments on Rabbits B, E, and O, at the end of the first absorption period, the loops were thoroughly washed out with physiological salt solution and the study of the absorption from these same loops was continued for a second period of like duration.

† The entire small intestine was used in this experiment.

V) and only 8.7 per cent of an equivalent quantity of hippurate. In Rabbits B, E, and O two loops were prepared and benzoate placed in one and hippurate in the other. At the end of the 30 minute period the solutions were removed and new solutions were introduced, benzoate being placed in the hippurate loop and hippurate in the benzoate loop. In these rabbits the same results were found during the second absorption period as were found in the first; *i.e.*, a more rapid absorption of benzoate than of hippurate. These experiments demonstrated that sodium hippurate was only slowly absorbed from loops which readily absorbed sodium benzoate.

## DISCUSSION.

Parenteral administration of hippuric acid resulted in a more rapid elimination of hippurate in the urine than enteral administration. We believe that this delayed excretion following the feeding of hippurate was due to two factors, the slow rate of absorption of unchanged hippurate and the hydrolysis of a large proportion of the hippurate to benzoic acid. Hydrolysis of hippuric acid would produce glycine as well as benzoic acid. However, this quantity of glycine (1 mol) was previously shown to have

little or no influence on the rate of synthesis of hippuric acid after benzoate administration unless an excess of other amino acids was present (8). This would explain the failure of the glycine formed by hydrolysis to increase appreciably the rate of excretion of hippuric acid after hippurate feeding. It would also explain the slight increases observed in some experiments in which hippurate was administered with an excess of amino acids other than glycine (Tables III and IV).

It is evident that absorption of benzoic acid from the intestine would occur more slowly after feeding hippurate than after feeding benzoate. This might explain Csonka's observation that benzoyl glycuronic acid appeared in the urine of a pig after the administration of benzoate but not after the administration of hippurate. If conjugation with glycuronic acid is a secondary defense it might not be needed after the feeding of hippurate because of the delayed entrance of benzoic acid into the blood stream.

## SUMMARY.

1. A study has been made of the rate of excretion of hippuric acid in rabbits during a 6 hour period after its oral administration.

2. The rate of excretion of hippuric acid was markedly increased by the administration of hippurate with glycine or with proteins which have a high content of preformed glycine.

3. The increased rate of excretion of hippuric acid after feeding hippurate and glycine was considered to represent an increased rate of synthesis of hippuric acid from the benzoic acid formed by the hydrolysis of hippuric acid in the intestine and an excess of preformed glycine.

4. Sodium hippurate was only slowly absorbed from loops of the small intestine which rapidly absorbed sodium benzoate.

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# BENZOYLATED AMINO ACIDS IN THE ANIMAL ORGANISM.

## II. THE HYDROLYSIS OF HIPPURIC ACID IN THE ALIMENTARY CANAL OF THE RABBIT.

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In the preceding paper (1) evidence was presented which suggested that ingested hippuric acid was partially hydrolyzed to benzoic acid in the alimentary canal of the rabbit. These experiments were continued in order to determine in which part of the alimentary tract hydrolysis occurred and whether or not the hydrolytic agent was an enzyme present in one of the digestive secretions. In addition, a brief study was made of the behavior of benzoyl alanine and benzoyl-α-aminoisobutyric acid following their oral administration.

### *1. The Hydrolysis of Hippuric Acid in Isolated Sections of the Alimentary Canal.*

Direct evidence that considerable splitting of hippuric acid might occur in the alimentary canal was obtained by the following procedure. A rabbit was killed and the stomach, the small intestine, the cecum, and the ascending colon were ligated, removed from the animal, and placed in beakers containing warm Locke's solution. Sodium hippurate was injected into each section and these were then incubated at 37°C. At the end of a 6 hour period each section was cut into small pieces and the resulting mixture was acidified and made into a dry powder by the addition of $CaSO_4$. Free benzoic acid in this material was determined by the method of Raiziss and Dubin (2). The results of these experiments are found in Table I. In no case was there formation of benzoic

acid in the small intestine. In five experiments on the stomach significant hydrolysis occurred only in one. However, there was considerable production of benzoic acid in all of the experiments on the cecum and ascending colon. In several of these experiments benzoic acid was isolated and identified by its melting point.

TABLE I.

*The Hydrolysis of Hippuric Acid to Benzoic Acid in Isolated Sections of the Alimentary Canal.*

| Experiment No. | Section of alimentary canal used. | Benzoic acid added as sodium hippurate. | Benzoic acid found. | | Incubation period. |
|---|---|---|---|---|---|
| | | *gm.* | *gm.* | *per cent* | *hrs.* |
| 1 | Stomach. | 0.682 | 0.006 | 0.9 | 5.5 |
| | Duodenum. | 0.409 | 0.000 | 0.0 | 5.5 |
| | Ileum. | 0.409 | 0.000 | 0.0 | 5.5 |
| | Cecum. | 0.818 | 0.201 | 25.0 | 5.5 |
| | Colon. | 0.409 | 0.052 | 12.7 | 5.5 |
| 2 | Stomach. | 0.682 | 0.005 | 0.7 | 7 |
| | Cecum. | 0.682 | 0.237 | 35.0 | 7 |
| | Colon. | 0.341 | 0.136 | 40.0 | 7 |
| 3 | Stomach. | 0.682 | 0.000 | 0.0 | 7 |
| | Cecum. | 0.682 | 0.213 | 31.0 | 7 |
| | Colon. | 0.682 | 0.051 | 16.0 | 7 |
| 4 | Stomach. | 0.341 | 0.023 | 7.0 | 6 |
| | Small intestine. | 0.426 | 0.000 | 0.0 | 6 |
| 5 | Cecum. | 0.682 | 0.460 | 68.0 | 6 |
| 6* | Stomach. | 2.180 | 0.490 | 22.0 | 6.5 |

* In this experiment, after placing a ligature at the pylorus, the hippurate solution was injected into the stomach. The rabbit was under urethane anesthesia during the course of the experiment.

### 2. *The Absorption of Sodium Benzoate from the Large Intestine.*

Since hydrolysis occurred only in the large intestine it would follow that benzoate must be readily absorbed from the large intestine inasmuch as ingested hippurate may be quantitatively recovered from the urine. Sodium benzoate was shown to be

readily absorbed from the cecum by the following experiment. A rabbit was anesthetized and a short abdominal incision was made along the median line. A solution containing 2.2 gm. of sodium benzoate was injected into the cecum and the point of injection was carefully ligated to prevent the escape of the benzoate from the cecum into the peritoneal cavity. After 6 hours the urine was collected and analyzed for free and combined benzoic acid. 0.060 gm. of free benzoic acid and 0.588 gm. of combined benzoic acid were found. These values were similar to those obtained after the oral administration of benzoate to rabbits (3).

*3. The Hydrolysis of Hippuric Acid by Histozyme.*

The hydrolysis of hippuric acid might have resulted from the action of an enzyme secreted by the digestive tract or from the action of microorganisms present in the alimentary canal. Since the benzoyl linkage in hippuric acid is similar to the peptide linkage, it was theoretically possible that one of the proteolytic enzymes, pepsin, trypsin, or erepsin might act on hippuric acid. However, neither trypsin (4, 5) nor erepsin (6, 7) hydrolyzes hippuric acid. The following experiment demonstrated that pepsin was likewise unable to hydrolyze this compound. An active preparation of pepsin which readily digested fibrin was incubated with 0.5 gm. of hippuric acid in 0.2 per cent HCl for 7 days. At the end of this incubation period the solution was analyzed for free benzoic acid with negative results.

Schmiedeberg (8) found an enzyme, histozyme, in dog liver and pig kidney which could hydrolyze hippuric acid into benzoic acid and glycine. Minkowski (9) was unable to find histozyme in the liver, muscles, or blood of rabbits. Van de Velde and Stokvis (10) also reported that histozyme was not present in the blood or kidneys of rabbits.

In order to determine whether this enzyme was present in the walls of the alimentary canal of the rabbit, preparations of the stomach and intestines were tested for hydrolytic action on hippuric acid. These preparations were made according to the procedure of Mutch (11). For comparison a preparation from fresh pig kidneys was made at the same time. It was evident from the following experiment that an active histozyme was present in the preparation from the pig kidneys. Flasks containing

sodium hippurate (equivalent to 0.068 gm. of benzoic acid), 0.2 gm. of the powdered tissue, and 5 cc. of toluene were incubated at 37°C. for from 1 to 7 days. The results are shown in Table II and are similar to those obtained by Mutch. However, preparations of the stomach and of the large and small intestines of the rabbit, made in the same manner as the preparation from pig kidneys, showed no hydrolytic action on hippuric acid. Glycerol extracts, 50 per cent alcoholic extracts, and minced fresh tissue likewise failed to split hippuric acid. These results were in agreement with the previous failures to find histozyme in the tissues of the rabbit.

There still remained the possibility that the observed hydrolysis of hippuric acid was due to an intracellular histozyme in certain microorganisms in the intestinal tract. This seemed quite prob-

TABLE II.

*The Hydrolysis of Hippuric Acid* by Histozyme from Pig Kidneys.*

| Incubation period. | Hippuric acid hydrolyzed. | |
|---|---|---|
| *hrs.* | *gm.* | *per cent* |
| 24 | 0.006 | 6 |
| 72 | 0.050 | 50 |
| 144 | 0.092 | 92 |
| 144 (Boiled.) | 0.000 | 0 |

* Solutions of sodium hippurate (equivalent to 0.1 gm. of hippuric acid) were used.

able from the fact that hydrolysis was found in the large intestine and not in the small intestine. Further evidence that this was the case was obtained in a number of experiments by using the following procedure. A sterile tube of cooked meat (12) was inoculated with material from the cecum of a rabbit. This culture was incubated for 24 hours and a loopful was transferred to a second tube. After 24 hours a loopful was transferred from the second to a third tube to which sodium hippurate had been added. The presence of free benzoic acid in this tube was shown by the following qualitative test after an incubation period of 72 hours. The contents of the tube were acidified and then extracted with toluene. The toluene was in turn extracted with dilute sodium hydroxide. This solution was acidified and extracted with ether. On evaporation of the ether and heating the residue benzoic acid

was sublimed. No trace of benzoic acid was produced in control tubes by bacterial action on the proteins of the meat. It was assumed that this hydrolysis of hippuric acid to benzoic acid was due to a bacterial histozyme and not to a histozyme secreted by the intestinal wall since the original loopful of material had been enormously diluted by transferring the cultures. The result of this experiment demonstrated that intestinal bacteria or molds may split hippuric acid.

*4. The Behavior of Benzoyl Alanine Following Its Oral Administration.*

Since benzoylated amino acids are eliminated unchanged after parenteral administration the presence of hippuric acid in the urine following the enteral administration of benzoyl alanine would be a positive demonstration of the intestinal hydrolysis of that substance. Hippuric acid was isolated from urine and identified by its crystalline appearance, melting point, and nitrogen content after feeding a rabbit *dl*-benzoyl alanine (as the sodium salt). Considerable difficulty was encountered in separating the hippuric acid from benzoyl alanine which was also present in the urine. The two compounds differ very little in solubility and structure and there was a tendency for them to crystallize together. This precipitate which was still obtained after three recrystallizations melted at 149–150°C. and contained 7.42 per cent nitrogen. An equimolecular mixture of hippuric acid and benzoyl alanine melted at 148–149°C. and contained 7.45 per cent nitrogen. A fourth crystallization gave the usual mixed product and in addition separate crystals which were easily removed and identified as crystals of hippuric acid.

Benzoyl alanine was hydrolyzed to benzoic acid in the cecum of the rabbit in experiments similar to those reported in Table I. The experimental substances (as sodium salts, equivalent to 0.170 gm. of benzoic acid) were injected into isolated sections of the cecum. After an incubation period of 6 hours the contents of the sections were acidified and made into a dry powder with $CaSO_4$. This material was tested qualitatively for free benzoic acid as described above. The results (Table III) were in agreement with the observation of Mutch (11) that *d*-benzoyl alanine was hydrolyzed by histozyme.

### *5. The Behavior of Benzoyl-α-Aminoisobutyric Acid Following Its Oral Administration.*

Benzoyl-α-aminoisobutyric acid was isolated from the urine following its oral administration (2.5 gm. as the sodium salt). The absence of extra hippuric acid in this urine indicated that there had been no appreciable intestinal hydrolysis of this substance. Benzoyl-α-aminoisobutyric acid was not hydrolyzed, except in traces, in experiments on the isolated cecum (Table III), and it was not hydrolyzed by histozyme from pig kidneys. Smorodinzew (13) likewise reported that this particular benzoylated amino acid was not hydrolyzed by histozyme.

TABLE III.

*The Hydrolysis of Benzoylated Amino Acids in Isolated Sections of the Large Intestine.**

| Experiment No. | Benzoylated amino acid. | Benzoic acid found. |
|---|---|---|
| 1 | Hippuric acid. | ++ |
| | Benzoyl alanine. | + |
| | Benzoyl-α-aminoisobutyric acid. | Trace. |
| 2 | Hippuric acid. | ++ |
| | Benzoyl alanine. | ++ |
| | Benzoyl-α-aminoisobutyric acid. | Trace. |
| 3 | Hippuric acid. | ++ |
| | Benzoyl alanine. | + |
| | Benzoyl-α-aminoisobutyric acid. | − |

* These benzoylated amino acids were injected into isolated sections of the cecum of a rabbit. After an incubation period of 6 hours the contents of the sections were tested qualitatively for free benzoic acid.

### DISCUSSION.

The experiments reported in this paper support the conclusions reached in the preceding paper that ingested hippuric acid was largely hydrolyzed before absorption from the alimentary canal of the rabbit. This hydrolysis occurred in the large intestine and was due to a bacterial histozyme, an enzyme having the function of splitting off benzoic acid from some benzoylated amino acids.

Although no attempt was made to determine which organism was responsible for the hydrolysis, it should be noted that hippuric acid may be hydrolyzed by many common microorganisms, such as *Staphylococcus albus* and *aureus* and streptococcus (14) and molds in the *Aspergillus* and *Penicillium* groups (15, 16). Stapp (17) found 35 species of microorganisms which could utilize hippuric acid or urea as the sole source of organic food material.

According to Smorodinzew (13) histozyme is an amidase, differing from pepsin, trypsin, and erepsin in that it hydrolyzes acyl derivatives of amino acids. Ando (18) found hippuric acid in the urine of rabbits following the feeding of cinnamoyl tyrosine but not following its subcutaneous injection. Shiple and Sherwin (19) found phenaceturic acid in the urine of a dog following the feeding of phenylacetyl glutamine and phenylacetyl ornithine although these substances were eliminated unchanged following parenteral administration. Our results make it seem probable that these acyl derivatives were hydrolyzed by a bacterial histozyme in the alimentary tract. Only those acyl derivatives of amino acids would be hydrolyzed which are acted upon by histozyme and which are absorbed so slowly that they pass on into the large intestine. Some, such as benzoyl-α-aminoisobutyric acid, are not split by histozyme and others are probably absorbed unchanged from the small intestine and thus escape hydrolysis. Experiments are in progress on the rate of absorption of some of these compounds from the small intestine.

## SUMMARY.

1. Hippuric acid and benzoyl alanine were found to be hydrolyzed in the large intestine of the rabbit, probably by an intracellular histozyme present in certain intestinal microorganisms.

2. Benzoyl-α-aminoisobutyric acid, which is not acted upon by histozyme, was not hydrolyzed in the large intestine of the rabbit.

3. Hippuric acid was isolated from the urine of rabbits after the oral administration of benzoyl alanine but not after the administration of benzoyl-α-aminoisobutyric acid.

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# THE FATE OF SUGAR IN THE ANIMAL BODY.

## I. THE RATE OF ABSORPTION OF HEXOSES AND PENTOSES FROM THE INTESTINAL TRACT.

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### I.

### INTRODUCTION.

Without an accurate knowledge of the laws of intestinal absorption it would seem impossible to follow the fate of sugar in the animal organism on a quantitative basis. For convenience, the fate of sugar in the animal body may be divided into four steps: first, the absorption from the intestinal tract; second, the passage through the blood and occasionally elimination in

the urine; third, the penetration into the tissues;[1] and fourth, the disposal in the tissues. The first and second steps are comparatively easy to follow quantitatively and form the content of this paper. It was originally intended to investigate only the fate of glucose. The other sugars as galactose, mannose, fructose, and pentoses were later on included, partly because some of them are frequently ingested with our food and are also physiological cell constituents, partly because it was found that they serve as an excellent illustration of the selective permeability of the intestinal membrane.

## II.

### *A Method for the Quantitative Study of Intestinal Absorption.*

Hitherto, most of the experiments on intestinal absorption have been performed on isolated intestinal loops of dogs, cats, or rabbits and generally one of two principles was followed. Either an acute experiment was made, which involved narcosis of the animal, a laparotomy, and the tying off of the desired portion of the intestine or a Vella fistula was established in a preceding operation. Both methods have their merits and demerits which need not be discussed here. Their chief disadvantage is that by working only on a part of the intestine, they do not allow the study of the mechanism of absorption of the whole intestinal tract as a physiological unity.

For our purpose, a method had to be devised, which would allow of estimating quantitatively the absorbing capacity of the whole intestine and which would permit of basing the results on a unit of body weight. Since only small laboratory animals can be obtained in sufficient number and of the desired uniformity of stock, age, and nutritional condition, the method was worked out on rats. The principle is briefly as follows: A known amount of the substance under investigation is fed by stomach tube. After a given time, the rats are killed and the amount of substance remaining in the intestines is determined quantitatively. The difference between the amount fed and the amount recovered from

[1] While this paper was in press the permeability of liver and muscles for hexoses and pentoses has been determined (Cori and Goltz, 1925, *a*).

the whole intestinal tract is then the amount of substance absorbed. The method is described in detail below.

Male and female albino rats of an inbred strain, originally derived from The Wistar Institute, Philadelphia, were used. They received a basal ration which was composed as follows: Ground scratch feed, 24 per cent; ground wheat, 20 per cent; ground fish, 20 per cent; milk powder, 20 per cent; lard, 15 per cent; cod liver oil, 1 per cent.[2] In addition, they received twice a week, some fresh vegetables. The growth of the rats maintained on this diet was entirely normal.

Rats between 2 and 3 months of age, weighing from 120 to 180 gm. were found most suitable. They were weighed 48 hours prior to the experiment and each rat was placed in a small wire screen cage. The feces dropped through the wire screen and were not accessible to the animals. Solid food was withheld throughout the 48 hours, but water was left at their disposal. After 48 hours, the rats were again weighed and the solution under investigation was fed by stomach tube. Urethral catheters Nos. 4 and 5 were very satisfactory for this purpose. These catheters soften when they are plunged for a moment into boiling water without making them permeable to liquids. A small mouthpiece served as a protection and a mark indicated the depth to which the catheter had to be introduced in order to reach the stomach. As a rule, the rats could be fed without causing excitement or inflicting pain. The only point that sometimes offered resistance was the diaphragmatic portion of the esophagus. If the catheter could not be introduced beyond this point while the rats were held in a ventral position, they were turned in a dorsal position and stretched. The catheter slides then easily into the stomach. A hypodermic needle was tightly fitted into the outside end of the catheter and the fluid was introduced by attaching a syringe to this needle. Preference was given to the Record type of syringe which has a metal plunger rather than the all glass syringes of the Luer type. Generally, between 1.25 to 2.5 cc. of a 25 to 80 per cent sugar solution, warmed up to 40°C., were given. Larger amounts of sugar solution of such a high percentage were found disadvantageous, since they sometimes caused diarrhea. After the fluid was delivered the catheter with the syringe still attached was quickly withdrawn from the esophagus. The very small diameter of the catheter prevents the escape of fluid that is still located in it and any liquid that adheres to the outside of the catheter is wiped off on the wall of the esophagus.

For reasons to be advanced later, it was not found necessary to give a constant amount of sugar per unit of body weight. This simplified the method to a great extent and allowed feeding the sugar with a high degree of accuracy. If each rat had to be given an amount of sugar according to

[2] This ration was furnished by Mr. C. Congdon from the Department of Nutritional Research of Park and Pollard Co.

weight, one would have to introduce different amounts of sugar solution into each rat, which would involve a rather high error in the amounts of fluid measured. It is also difficult to set the plunger with the desired degree of accuracy at a certain graduation mark of the syringe. This was avoided by introducing special stops into the syringe against which the plunger could firmly be brought to rest.

On each experimental day a larger number of rats received the same amount of sugar solution. The amount of sugar thus introduced was determined by delivering into a volumetric flask the same amount of sugar solution under exactly the same conditions as when the rats were fed. Even if the same sugar solution was used on subsequent days it was found necessary to determine anew the amount of sugar delivered. The rats were killed at hourly intervals after the sugar administration. Any rats that showed diarrhea subsequent to the sugar feeding were discarded. The abdominal cavity was opened and ligatures were placed around the esophagus and the rectum. The stomach, small intestine, and the whole large intestine were carefully detached from the mesentery and placed in a beaker. The whole intestinal tract was cut open, the instruments used carefully rinsed off, and the intestine washed out with successive portions of hot distilled water. Generally 500 cc. of water were used and the washings decanted into a volumetric flask. The interfering substances in the intestinal washings were precipitated with Merck's colloidal iron on the addition of a small amount of sodium sulfate. The flask was cooled, made up to the mark, well mixed, and the contents filtered. Aliquot parts of the clear filtrate were then taken for analysis but not until almost the whole amount of fluid had filtered through.

The following control experiments were performed in order to test the accuracy of the different steps involved in this method.

*1. The Determination of the Amount of Sugar Fed to the Rats.*—2 cc. of an approximately 50 per cent glucose solution at 40°C. were drawn up into the syringe, the syringe attached to the catheter, and the fluid delivered into a 1000 cc. volumetric flask. Another 2 cc. were delivered into a second flask. Both flasks were made up to the mark and aliquot parts taken for sugar determinations. The first flask contained 1.097 gm. glucose, the second flask 1.10 gm. glucose.

*2. The Amount of Sugar Adhering to the Outside of the Catheter after Withdrawing It from the Esophagus.*—After feeding rats with 2.5 cc. of a 50 per cent glucose solution, the outside of the withdrawn catheter was carefully rinsed off. 2.1 mg. glucose were recovered as an average of four experiments.

*3. The Amount of Sugar Recovered from the Intestine When the Rats Were Killed Immediately after the Sugar Feeding.*—Three rats, fasted previously for 48 hours, received 2.5 cc. each of a 50 per cent glucose solution and were killed immediately. From 99.4 to 99.8 per cent glucose was recovered.

4. *The Amount of Reducing Substances Normally Present in the Intestine of Rats That Were Fasted for 48 Hours.*—In three experiments, using the micro method of Hagedorn and Jensen, 13.9, 9.0, and 12.7 mg. reducing sub-

stances were found respectively. This amount, when compared with the amount of sugar fed, seemed so small that it was neglected in the calculations.

*5. How Soon after the Sugar Feeding Does Absorption Begin?*—Since part of the sugar solution is forced during the injection, into the small intestine, the absorption begins at once. A rat killed 2 minutes after sugar feeding already showed half of the small intestine filled with sugar solution.

*6. Is There Any Loss of Sugar From the Intestine Due to Bacterial Action?*—This was determined for each sugar in the following way. Rats fasted for 48 hours were killed immediately after feeding 2.5 cc. of a 50 per cent sugar solution and 2.5 cc. of distilled water. Ligatures were made around the esophagus and the lowest portion of the ileum. The stomach and small intestine were removed and the contents evenly distributed by gentle massage. After incubating for 3 to 5 hours at 37°C., stomach and small intestine were cut open and the sugar content was determined in the manner already described. The losses of sugar due to bacterial action were so small that they were neglected in the calculations.

The following sugars were used in our experiments.

| | $[\alpha]_D^{20}$ |
|---|---|
| *d*-Glucose Merck | 52.45 |
| " Pfanstiehl | 52.5 |
| *d*-Galactose Kahlbaum | 79.8 |
| " Pfanstiehl | 80.0 |
| *d*-Mannose " | 14.25 |
| *d*-Fructose " | −91.66 |
| *l*-Arabinose " | 104.5 |
| *l*-Xylose " | 18.5 |

25, 50, and 80 per cent solutions from glucose and 50 per cent solutions from the other sugars were fed. The sugars were brought into solution in distilled water at least 24 hours before they were used, so that any possible complication due to a displacement of the usual $\alpha$ and $\beta$ equilibrium of the sugars might be avoided. For all sugar determinations Bertrand's method was used. The permanganate used for the titration was standardized against C.P. glucose. For mannose, arabinose, and xylose which are not recorded in the original Bertrand table, the copper equivalents for the range of 10 to 20 mg. sugar were especially determined. For blood sugar Hagedorn and Jensen's (1923) method was used.

III.

*The Loss of Body Weight of Rats Fasted for 48 Hours.*

The loss of body weight, expressed in per cent of the body weight at the beginning of the fasting period, is recorded in Tables I to VIII. It was 12.92 per cent as an average of 124 observations, the maximum being 16.02 and the minimum 8.34 per cent. Rats that did not show a loss of weight falling within this range were considered abnormal and were not used. The variation in the loss of body weight is in part due to an individual variability of the rats, in part to irregular food intake prior to the fasting period, and also to some extent to the uncertainty with which the original body weight of the rats can be determined. Rats which are not fasted may change their weight by 1 to 2 gm. in the course of a very short time by the loss of feces and urine. All calculations were based on the weight of the rats after fasting for 48 hours.

IV.

*The Relation of the Absorbing Surface of the Intestine to the Body Weight.*

The amount of a given substance that is absorbed should be proportional to the absorbing surface of the intestinal tract. Since the surface of the intestine will vary with the body weight, larger animals will have a greater intestinal surface and will absorb more than smaller ones. In order to use animals of different weight one would have to show that the absorbing surface is strictly proportional to the body weight or in other words that the quotient $\frac{\text{intestinal surface}}{\text{body weight}}$ or $\frac{\text{amount absorbed}}{\text{body weight}}$ is a constant. Our experiments reveal that within the range of body weights investigated, namely between 100 and 180 gm., both sexes show a proportionality between the amount absorbed and hence between intestinal surface and body weight. An example taken from Table II might illustrate this. A female rat weighing 117.7 gm. absorbed in 1 hour 0.219 gm. glucose or $\frac{0.219 \times 100}{117.7} = 0.186$ gm. glucose per 100 gm. of body weight. A male rat weighing 173.7 gm. absorbed in 1 hour 0.320 gm. glucose or $\frac{0.320 \times 100}{173.7} =$

0.184 gm. glucose per 100 gm. of body weight. It should be noted that this proportionality between the amount absorbed and the body weight was found in rats that were still in a period of active growth. It may not be valid for fully grown rats, where variations in the fat depots and other factors might play a rôle.

## V.

## *The Absorption Coefficient.*

On the basis of the preceding section the mathematical expression of intestinal absorption is very simple. The absorption coefficient ($C$) is the amount of substance absorbed per 100 gm. of body weight in 1 hour and can be represented by the following formula.

$$C = \frac{A \times 100}{W \times T} \qquad (1)$$

where $A$ is the total amount of substance absorbed in a given period, $W$ the weight of the rat after fasting for 48 hours and $T$ the length of the period in hours.

TABLE I.

*The Absorption of 25 Per cent Glucose Solution.*

| Loss of original body weight in 48 hrs. | Blood sugar. | Glucose fed per 100 gm. of body weight. | Glucose absorbed per 100 gm. of body weight in 1 hr. (Absorption coefficient.) | Remarks. |
|---|---|---|---|---|
| *per cent* | *per cent* | *gm.* | *gm.* | |
| 13.24 | 0.162 | 0.399 | 0.172 (max. 0.203) (min. 0.152) | Absorption period 1 hour. Average of six rats. |

TABLE II.

*The Absorption of 50 Per Cent Glucose Solution.*

The amount of fluid introduced was 2.5 cc., with the exception of two rats marked with an asterisk, which received 1.25 cc.

| Sex. | Body weight after fasting 48 hrs. | Loss of original body weight in 48 hrs. | Blood sugar. | Glucose fed per 100 gm. of body weight. | Total amount of glucose absorbed. | Glucose absorbed per 100 gm. of body weight in 1 hr. (Absorption coefficient.) |
|---|---|---|---|---|---|---|
| **Absorption period 1 hr.** | | | | | | |
| | *gm.* | *per cent* | *per cent* | *gm.* | *gm.* | *gm.* |
| M. | 169.5 | 13.16 | 0.204 | 0.649 | 0.334 | 0.197 |
| " | 170.2 | 13.38 | 0.171 | 0.646 | 0.330 | 0.194 |
| " | 173.7 | 12.53 | 0.168 | 0.659 | 0.320 | 0.184 |
| " | 151.8 | 13.15 | 0.170 | 0.754 | 0.298 | 0.196 |
| " | 160.1 | 11.87 | 0.195 | 0.715 | 0.311 | 0.194 |
| " | 118.1 | 13.28 | 0.168 | 0.442* | 0.241 | 0.204 |
| F. | 148.9 | 14.52 | 0.186 | 0.772 | 0.316 | 0.212 |
| " | 117.7 | | 0.149 | 0.469* | 0.219 | 0.186 |
| Average..... | | 13.12 | 0.176 | 0.638 | | 0.196 |
| **Absorption period 2 hrs.** | | | | | | |
| M. | 157.8 | 12.81 | 0.177 | 0.713 | 0.478 | 0.151 |
| " | 155.0 | 14.78 | 0.240 | 0.726 | 0.546 | 0.176 |
| " | 159.4 | 12.00 | 0.261 | 0.706 | 0.450 | 0.141 |
| " | 149.6 | 12.76 | 0.219 | 0.715 | 0.540 | 0.180 |
| " | 158.2 | 12.05 | 0.213 | 0.676 | 0.431 | 0.136 |
| " | 155.2 | 12.01 | 0.214 | 0.747 | 0.568 | 0.183 |
| Average..... | | 12.73 | 0.220 | 0.713 | | 0.161 |
| **Absorption period 3 hrs.** | | | | | | |
| M. | 137.5 | 13.19 | 0.226 | 0.834 | 0.744 | 0.180 |
| " | 144.8 | 13.24 | 0.233 | 0.801 | 0.692 | 0.159 |
| " | 158.8 | 16.02 | 0.231 | 0.705 | 1.010 | 0.212 |
| " | 144.6 | 14.43 | 0.255 | 0.774 | 0.760 | 0.180 |
| " | 152.7 | 12.14 | 0.193 | 0.733 | 0.734 | 0.160 |
| Average..... | | 13.80 | 0.227 | 0.769 | | 0.178 |
| Average of 1, 2, and 3 hr. absorption periods..... | | | | | | 0.178 |

TABLE III.

*The Absorption of 80 Per Cent Glucose Solution.*

| Loss of original body weight in 48 hrs. | Blood sugar. | Glucose fed per 100 gm. of body weight. | Glucose absorbed per 100 gm. of body weight in 1 hr. (Absorption coefficient.) | Remarks. |
|---|---|---|---|---|
| *per cent* | *per cent* | *gm.* | *gm.* | |
| 11.57 | 0.157 | 0.819 | 0.143 (max. 0.183) (min. 0.126) | Absorption period 1 hr. Average of seven rats. |
| 12.35 | 0.187 | 1.082 | 0.159 (max. 0.214) (min. 0.131) | Absorption period 2 hrs. Average of six rats. |
| 11.15 | 0.167 | 1.253 | 0.153 (max. 0.180) (min. 0.122) | Absorption period 3 hrs. Average of five rats. |
| 12.62 | 0.162 | 1.323 | 0.171 (max. 0.219) (min. 0.134) | Absorption period 4 hrs. Average of five rats. |
| 13.92 | 0.201 | 1.459 | 0.184 (max. 0.203) (min. 0.170) | Absorption period 5 hrs. Average of five rats. |
| Average of 1, 2, 3, 4, and 5 hr. absorption periods.. | | | 0.162 | |

TABLE IV.

*The Absorption of 50 Per Cent Galactose Solution.*

| Loss of original body weight in 48 hrs. | Blood sugar. | Galatose fed per 100 gm. of body weight. | Galatose absorbed per 100 gm. of body weight in 1 hr. (Absorption coefficient.) | Remarks. |
|---|---|---|---|---|
| *per cent* | *per cent* | *gm.* | *gm.* | |
| 14.30 | 0.298 | 0.791 | 0.220 (max. 0.247) (min. 0.190) | Absorption period 1 hr. Average of five rats. |
| 14.51 | 0.325 | 0.905 | 0.186 (max. 0.201) (min. 0.172) | Absorption period 2 hrs. Average of four rats. |
| 12.32 | 0.361 | 0.946 | 0.184 (max. 0.196) (min. 0.177) | Absorption period 3 hrs. Average of five rats. |
| Average of 1, 2, and 3 hr. absorption periods..... | | | 0.196 | |

TABLE V.

*The Absorption of 50 Per Cent Fructose Solution.*

| Loss of original body weight in 48 hrs. | Blood sugar. | Fructose fed per 100 gm. of body weight. | Fructose absorbed per 100 gm. of body weight in 1 hr. (Absorption coefficient.) | Remarks. |
|---|---|---|---|---|
| *per cent* | *per cent* | *gm.* | *gm.* | |
| 12.18 | 0.144 | 0.716 | 0.075 (max. 0.096) (min. 0.059) | Absorption period 1 hr. Average of five rats. |
| 12.56 | 0.130 | 0.485 | 0.073 (max. 0 090) (min. 0.063) | Absorption period 2 hrs. Average of four rats. |
| 11.82 | 0.150 | 0.583 | 0.079 (max. 0.097) (min. 0.063) | Absorption period 3 hrs. Average of four rats. |
| 12.46 | 0.177 | 0.601 | 0.077 (max. 0.091) (min. 0.065) | Absorption period 4 hrs. Average of four rats. |
| 13.43 | 0.176 | 0.708 | 0.080 (max. 0.087) (min. 0.071) | Absorption period 5 hrs. Average of four rats. |
| Average of 1, 2, 3, 4, and 5 hr. absorption periods.. | | | 0.077 | |

TABLE VI.

*The Absorption of 50 Per Cent Mannose Solution.*

| Loss of original body weight in 48 hrs. | Blood sugar. | Mannose fed per 100 gm. of body weight. | Mannose absorbed per 100 gm. of body weight in 1 hr. (Absorption coefficient.) | Remarks. |
|---|---|---|---|---|
| *per cent* | *per cent* | *gm.* | *gm.* | |
| 13.66 | 0.106 | 0.851 | 0.033 (max. 0.036) (min. 0.028) | Absorption period 1 hr. Average of three rats. |
| 12.70 | 0.111 | 0.565 | 0.035 (max. 0.041) (min. 0.028) | Absorption period 2 hrs. Average of four rats. |
| 11.78 | 0.099 | 0.581 | 0.037 (max. 0.041) (min. 0.031) | Absorption period 3 hrs. Average of three rats. |
| 11.39 | 0.106 | 0.477 | 0.031 (max. 0.036) (min. 0.027) | Absorption period 4 hrs. Average of three rats. |
| 12.83 | 0.112 | 0.538 | 0.036 (max. 0.038) (min. 0.034) | Absorption period 5 hrs. Average of two rats. |
| Average of 1, 2, 3, 4, and 5 hr. absorption periods.. | | | 0.034 | |

TABLE VII.

*The Absorption of 50 Per Cent Xylose Solution.*

| Loss of original body weight in 48 hrs. | Blood sugar. | Xylose fed per 100 gm. of body weight. | Xylose absorbed per 100 gm. of body weight in 1 hr. (Absorption coefficient.) | Remarks. |
|---|---|---|---|---|
| *per cent* | *per cent* | *gm.* | *gm.* | |
| 12.04 | 0.125 | 0.411 | 0.062 (max. 0.074) (min. 0.055) | Absorption period 1 hr. Average of six rats. |
| 13.72 | 0.136 | 0.404 | 0.024 (max. 0.028) (min. 0.020) | Absorption period 2 hrs. Average of three rats. |
| 14.26 | 0.115 | 0.396 | 0.028 (max. 0.035) (min. 0.025) | Absorption period 3 hrs. Average of four rats. |
| 14.67 | 0.134 | 0.408 | 0.032 (max. 0.041) (min. 0.026) | Absorption period 4 hrs. Average of three rats. |
| 13.87 | 0.118 | 0.396 | 0.028 (max. 0.033) (min. 0.025) | Absorption period 5 hrs. Average of three rats. |
| Average of 2, 3, 4, and 5 hr. absorption periods. . | | | 0.028 | |

TABLE VIII.

*The Absorption of 50 Per Cent Arabinose Solution.*

| Loss of original body weight in 48 hrs. | Blood sugar. | Arabinose fed per 100 gm. of body weight. | Arabinose absorbed per 100 gm. of body weight in 1 hr. (Absorption coefficient.) | Remarks. |
|---|---|---|---|---|
| *per cent* | *per cent* | *gm.* | *gm.* | |
| 14.13 | 0.095 | 0.509 | 0.016 (max. 0.022) (min. 0.012) | Absorption period 2 hrs. Average of eight rats. |

VI.

*The Graphic Representation of the Rate of Absorption of Sugars.*

When the amount of sugar that is absorbed per 100 gm. of body weight is plotted against time, a straight line is obtained, as is illustrated in Fig. 1. The observations show a maximal deviation from the straight lines of ± 10 per cent, which is as close an

agreement as can be expected from a biological method such as the one used for this work. Fig. 1 serves also as an illustration of the rate of absorption of the different sugars. The more the straight lines are slanting to the right, the slower is the rate of absorption and *vice versa*.

Arabinose has not been included in this figure, because not enough data for a graphic representation could be secured. This

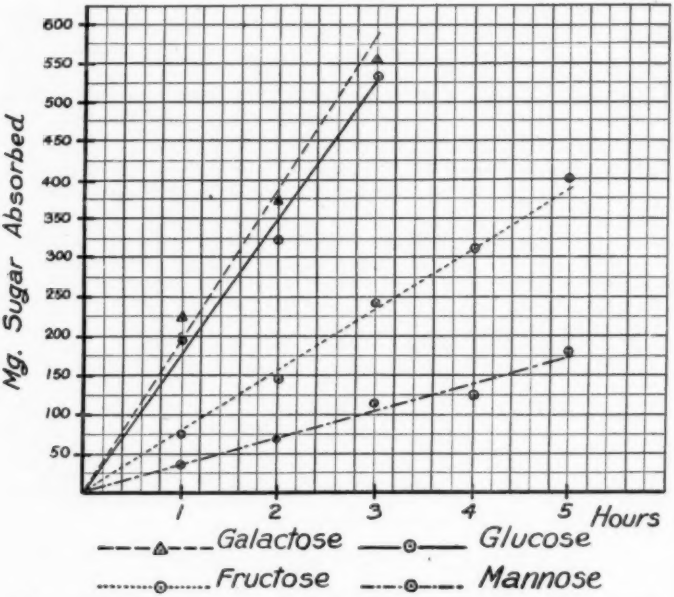


FIG. 1. Graphic representation of the rate of absorption of hexoses. The figures for the absorption coefficients in Tables II and IV to VI have been used for the construction of the straight lines.

sugar is absorbed so slowly that when the absorption is allowed to proceed over 2 hours, diarrhea developes in the majority of the rats. Xylose, on the other hand, differs from the rest of the sugars investigated since much more is absorbed in the 1st hour than in the following hours (see Table VII). From the 2nd to 5th hour the absorption of xylose follows a straight line in ac-

cordance with the other sugars. The reason for the higher rate of absorption in the 1st hour needs further investigation.

The fact that the absorption of sugar follows a straight line means that in 2 hours twice as much sugar is absorbed as in 1 hour and in 3 hours three times as much as in 1 hour and so on; in other words, the absorption proceeds at a constant rate. The amount and also the concentration of the sugar originally introduced into the intestine diminishes more and more the longer the absorption is allowed to proceed. Does this finally lead to a decrease of the rate of absorption? Or how far may the sugar present in the intestine diminish without causing a decrease in the rate of absorption? The straight lines in Fig. 1, with the exception of that of mannose, extend to a point where about 60 to 70 per cent of the sugar originally introduced has been absorbed. A calculation made from Table II may illustrate this for glucose. In the 3 hour period, as an average, 0.769 gm. glucose was fed and 0.534 gm. glucose was absorbed per 100 gm. of body weight. The difference between these two values, namely 0.235 gm. glucose, represents the amount of glucose that was still present in the intestine at the end of the 3 hour period. Up to this point, or when 69.5 per cent of the sugar originally introduced was absorbed, the rate of absorption had not diminished as can be seen from a comparison of the average absorption coefficient of this period with that of the preceding period. If individual experiments of the 3 hour period of Table II are examined one finds still smaller amounts of sugar remaining in the intestine, without a diminution of the rate of absorption. The third rat, for instance, received 0.705 gm. glucose and absorbed 0.636 gm. glucose per 100 gm. of the body weight. This corresponds to an absorption of 90.2 per cent of the glucose originally introduced. In spite of the fact that only 69 mg. glucose remained in the intestine of this rat, the rate of absorption had not diminished as can be seen from the absorption coefficient. Similar calculations can be made from Tables IV and V for galactose and fructose. It can, therefore, be concluded that these sugars and probably also the other sugars investigated are absorbed up to the point of their complete disappearance from the intestine at a constant rate. From this it follows that the rate of absorption must be independent of both the absolute amount and the concentration of sugar present in the intestine,

which means that neither an increase nor a decrease of these two factors can change the rate of absorption, or that there is only one rate of absorption for each particular sugar. These conclusions are the logical consequence of the fact that the absorption of sugar follows a straight line. Other experimental evidence points in the same direction.

If in Tables I to VIII,[3] the amounts of sugar given per 100 gm. of body weight are compared with the absorption coefficients it will be noted that the rate of absorption is independent of the absolute amount of sugar fed. An example from Table III may serve as an illustration. Thus a rat weighing 174.4 gm. received 0.496 gm. glucose per 100 gm. of body weight and absorbed 0.130 gm. glucose per 100 gm. of body weight per hour. Another rat also weighing 174.4 gm. received 1.037 gm. glucose per 100 gm. of body weight or twice as much and absorbed 0.126 gm. glucose per 100 gm. of body weight per hour. This means that the rate of absorption cannot be increased by increasing the amount of sugar fed. This example serves also as an illustration that the rate of absorption is within certain limits independent of the absolute amount of fluid introduced, since the first rat received 1.25 cc. of an 80 per cent glucose solution, while the second rat received 2.5 cc. of an 80 per cent glucose solution.

The sugar solution does not maintain its original concentration when it is introduced into the alimentary canal of the rats. Even in the stomach a dilution mechanism sets in and a further diminution of the sugar concentration occurs in the small intestine. Since the rate of absorption is independent of the constant diminution of the sugar concentration that is taking place during absorption, it should also be independent of the sugar concentration originally introduced. This has been tested experimentally for glucose solutions ranging from 25 to 80 per cent. The data are summarized in Table IX.

The average figures of Table IX agree within the limits of error of the method and are considered as additional evidence that the rate of absorption of sugar is independent of the actual sugar concentration in the intestine. The experiments recorded in Table

[3] Due to lack of space Tables I and III to VIII had to be represented in a condensed form. For this reason the figures for the example given below will not be found in the tables referred to.

IX were also undertaken in order to see which sugar concentration would be best suited for a comparative study of the rate of absorption of different sugars. If the absorption coefficients of the 1 hour period in Table IX are compared with each other it will be noted that less sugar is absorbed from the 80 per cent solution than from the 25 and 50 per cent solution. This anomaly may be due to the high viscosity of the 80 per cent solution, since more sugar is absorbed in the subsequent periods where a dilution and consequently a decrease in the viscosity has occurred. The 50 per cent solution has the advantage over the 25 per cent solution that enough sugar can be introduced to let the absorption proceed for at least 3 hours. Absorption periods of several hours are desirable for a graphic representation. In order to use the 25 per cent solution one would have to introduce too large volumes of fluid, which are apt to cause diarrhea. For these reasons a 50 per cent sugar solution has been chosen for comparative studies.

TABLE IX.

*The Influence of a 25, 50, and 80 Per Cent Glucose Solution on the Rate of Absorption (see Tables I, II, and III).*

| Length of absorption period. | Absorption coefficients for glucose solutions of: | | |
|---|---|---|---|
| | 25 per cent. | 50 per cent. | 80 per cent. |
| hrs. | | | |
| 1 | 0.172 | 0.196 | 0.143 |
| 2 | | 0.161 | 0.159 |
| 3 | | 0.178 | 0.153 |
| 4 | | | 0.171 |
| 5 | | | 0.184 |
| Average..... | 0.172 | 0.178 | 0.162 |

VII.

*The Comparison of the Rate of Absorption of Hexoses and Pentoses.*

Table X contains the average absorption coefficients of the different sugars investigated. The most important factor that determines the rate of absorption of a particular sugar seems to be the structure of its molecule. A relatively slight change in the sugar molecule affects the rate of absorption very markedly.

The keto-sugar fructose is absorbed with a rate less than one-half of that of glucose. A stereoisomeric change from glucose to mannose reduces the rate of absorption to one-fifth. The pentoses are absorbed at still slower rates. All this indicates that the intestinal membrane is possessed of a high degree of selective permeability for sugars.

TABLE X.

*Comparison of the Rate of Absorption of 50 Per Cent Solution of Hexoses and Pentoses.*

| Type of sugar. | Average blood sugar. | Average absorption coefficient. | Ratio (glucose = 100). |
|---|---|---|---|
| | *per cent* | *gm.* | |
| *d*-Galactose | 0.328 | 0.196 | 110 |
| *d*-Glucose | 0.207 | 0.178 | 100 |
| *d*-Fructose | 0.155 | 0.077 | 43 |
| *d*-Mannose | 0.106 | 0.034 | 19 |
| *l*-Xylose | 0.125 | 0.028 | 15 |
| *l*-Arabinose | 0.095 | 0.016 | 9 |

VIII.

*The Dilution Mechanism in the Stomach and Intestine and the Water Content of Blood and Tissues during the Absorption of a Hypertonic Sugar Solution.*

It was noted that the rats showed a greatly distended stomach, when examined 1 to 3 hours after sugar feeding. This suggested that in the stomach water was attracted by the hypertonic solution. An examination of the sugar concentration in the stomach in different time intervals after giving 2.5 cc. of an 80 per cent glucose solution showed:

| Hrs. | 0 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| Per cent | 73.2 | 38.0 | 30.9 | 24.5 | 14.3 |

Since the stomach does not have a very marked absorbing capacity for sugar, the diminution of the sugar concentration in the stomach is mainly due to the excretion of water.

The sugar concentration in the duodenum and jejunum is always lower than in the stomach and falls off more and more in the lower parts of the small intestine. A rat weighing 191.6 gm.,

with a total length of the small intestine of 87 cm., showed the following sugar concentrations 3 hours after giving 2.5 cc. of an 80 per cent glucose solution. Stomach, 25.0 per cent; small intestine, 0 to 33 cm., 14.2 per cent; small intestine, 33 to 50 cm., almost empty; small intestine 50 to 87 cm., 2.65 per cent. The total amount of fluid recovered from stomach and small intestine was 5.1 cc. The diminution of the sugar concentration in the small intestine is partly due to the absorption of sugar, partly to the secretion of water and it is difficult to separate both factors. It is said that in the upper part of the small intestine relatively more sugar is absorbed than water, while the opposite is true in the lower part of the small intestine. This point has not been investigated in connection with our experiments.

TABLE XI.

*The Effect of the Absorption of a Hypertonic Glucose Solution on the Water Content of the Organs and the Blood.*

The rats, fasted previously for 48 hours, were killed in hourly intervals after giving 2.5 cc. of an 80 per cent glucose solution. The values of each period are an average of several experiments.

| Length of absorption period. | Water content of: | | | |
|---|---|---|---|---|
| | Blood. | Muscle. | Liver. | Skin. |
| *hrs.* | *per cent* | *per cent* | *per cent* | *per cent* |
| 0 (Controls.) | 78.21 | 75.48 | 69.26 | 53.31 |
| 1 | 77.23 | 74.90 | 70.06 | 45.58 |
| 2 | 78.47 | 74.45 | 69.95 | 46.95 |
| 3 | 78.77 | 74.69 | 70.55 | 46.24 |
| 4 | 78.28 | 74.04 | 70.09 | 45.98 |
| 5 | 78.47 | 74.53 | 69.80 | 45.05 |

It seemed of importance to know if the high sugar concentrations that were given to the rats caused any unphysiological conditions. The animals did not show signs of distress subsequent to the sugar feeding nor did the blood sugar rise abnormally high. However, there might have been an excessive dehydration of blood and tissues due to the secretion of water into the intestine. This would have been undoubtedly a serious disturbance. For these reasons the water content of the blood, liver, muscle, and skin has been examined in different time intervals after the sugar

feeding. The tissues were dried for 1 week at 97°C. in a constant temperature oven. The weighing bottles were then stoppered and allowed to cool in a $CaCl_2$ desiccator. Table XI shows the result of this examination. With the exception of the skin no significant changes occurred in the water content of the other tissues and of the blood. The skin serves as the principal water depot of the organism, while the vital organs are apparently protected against dehydration. During the absorption of a hypertonic sugar solution from the intestine, water is constantly withdrawn from the blood. Since the water content of the blood did not diminish, the mechanism of release of water from the skin must be very accurately adjusted and must be set in motion very rapidly. It may be concluded that the use of sugar solutions of high concentrations for absorption work does not result in abnormal conditions.

IX.

*The Sugar and Nitrogen Excretion in the Urine, the Blood Sugar Concentration, and the Relation of the Absorption of Sugar from the Intestine to the Disposal of Sugar in the Tissues.*

Table XII shows that a negligible amount of glucose is excreted in the urine, even if as much as 15 gm. per kilo are fed. The excretion, after the subtraction of the amount of reducing substances normally eliminated in the urine, amounts to 1 mg. of sugar per 100 gm. of body weight per hour. The nitrogen excretion of the sugar-fed rats is lower than that of the fasting controls, which corresponds to the well known sparing action of carbohydrate ingestion on the protein metabolism. Whether the small amount of reducing substances eliminated in the urine of fasting rats is actually sugar has not been determined.

The blood sugar concentration during absorption is dependent on three factors: the rate of absorption of sugar from the intestine; the rate of disposal of sugar in the tissues; the amount of sugar excreted in the urine. The relation between the blood sugar concentration and the rate of absorption of different sugars is shown in Table X. The slower the rate of absorption, the lower is the blood sugar concentration and *vice versa*. If the first and

the last factor are known an insight is gained into the rate of disposal of sugar in the tissues. The first factor can be considered as constant, since the rate of absorption proceeds as a straight line and since it is independent of the total amount and concentration of sugar present in the intestine. The excretion in the urine of other sugars than glucose has not been extensively enough studied. For glucose one can conclude that under the conditions of our experiments the rate of disposal in the tissues is parallel to the rate of absorption, since practically no sugar is excreted in the urine and since the blood sugar does not rise very high. The free sugar

TABLE XII.

The sugar and nitrogen excretion in the urine after giving:

(*a*) 2.5 cc. of water (controls).

(*b*) 2.5 cc. of an 80 per cent glucose solution (sugar-fed rats).

(*c*) The same as (*b*) plus 20 units of insulin subcutaneously (sugar-fed and insulinized rats).

All experiments were performed on the same group of rats. The animals were fasted for 48 hours previously. The analyses were made on the combined urine of the animals used for each experiment.

| No. of rats. | Combined body weight. | Length of absorption period. | Glucose fed per 100 gm. of body weight. | Nitrogen excreted per 100 gm. of body weight in 1 hr. | Sugar excreted per 100 gm. of body weight in 1 hr. | Remarks. |
|---|---|---|---|---|---|---|
| | *gm.* | *hrs.* | *gm.* | *mg.* | *mg.* | |
| 9 | 1212 | 5 | | 2.45 | 0.32 | Controls. |
| 6 | 872 | 5 | 1.471 | 2.33 | 1.36 | Sugar-fed rats. |
| 8 | 1162 | 5 | 1.498 | 2.31 | 0.71 | Sugar-fed and insulinized rats. |

concentration in the tissues is dependent on the blood sugar concentration. If the blood sugar would rise abnormally high, part of the glucose could be held by the tissues as such without being metabolized. At blood sugar concentrations such as were observed during the absorption of glucose, a retention of sugar in the free state by the tissues plays a minor rôle, so that all the glucose that is absorbed in a given period is also metabolized in that period. If the rate of disposal in the tissues would fall even slightly below the rate of absorption the blood sugar would rise, as can be seen from the following calculation. If we assume that the blood

volume of a rat weighing 100 gm. is 7 cc. and if we assume a blood sugar of 0.1 per cent, 7 mg. sugar would be held by the blood. During 1 hour 178 mg. glucose or twenty-five times as much enter the blood from the intestine and have also to leave the blood if the blood sugar concentration is to remain the same. If in 1 hour, 7 mg. less glucose would be metabolized than are absorbed, the blood sugar would rise to 0.2 per cent. If this would go on for another hour, the blood sugar would rise to 0.3 per cent and so forth, not considering the sugar excretion in the urine. If, however, the rate of disposal of glucose in the tissues is parallel to the rate of absorption, the blood sugar values of successive absorption periods should remain the same. In Table III, the absorption was allowed to proceed for 5 hours. The average blood sugar at the end of the 1st hour was 0.157 per cent, at the end of the 5th hour 0.201 per cent, which is an insignificant change on the basis of the calculation just given.

## X.

### DISCUSSION.

An outline of an investigation of the fate of sugar in the animal organism has been given in the introduction. The object of the experiments recorded in this paper was to study the intestinal absorption of sugars, particularly that of glucose, on a quantitative basis. For this purpose a method has been worked out, which allowed us to measure and express absorption in terms of unit of body weight and unit of time. One result of the use of this method was that the absorption of hexoses was found to follow a straight line. What can be learned from this fact in regard to the mechanism of absorption in general it would be too early to discuss, since it has not been investigated as to whether or not other non-electrolytes show the same behavior as sugars. Nor has the absorption of lipoid-soluble substances and of electrolytes been studied with this method. The results of previous investigators are not strictly comparable, since they are based on observations on isolated parts of the intestine. The study of absorption by the whole intestinal tract as a physiological unity and the possibility of constructing definite absorption curves for each substance may give results differing in many respects. For these reasons it seemed advisable to postpone a discussion of the

mechanism of absorption until these experiments have been performed.

Another result was that stereoisomeric sugars were found to be absorbed at different rates from the intestine. This is a confirmation of data to be found in the literature. Nagano (1902) used four dogs with a Vella fistula of the small intestine of 18 to 27 cm. in length. 30 cc. of 1 to 7.5 per cent sugar solutions were introduced and allowed to remain in contact with the intestine for 1 hour. On Dog 1 all sugars were tested in succession. The following order in the rate of absorption was found: galactose > glucose > fructose > mannose > xylose > arabinose. Similar results were obtained on the other dogs on which only some of the sugars just mentioned were tested. Hewitt (1924) made experiments on rabbits and cats. Approximately 2 per cent solutions of glucose, fructose, and galactose were used. In the series with rabbits ether narcosis was applied. The author mentions that the experiments were not strictly comparable since varying lengths of intestine were isolated in the different animals and since the sugars were apparently not tested in succession on the same intestinal loop. He merely draws the conclusion that glucose is more readily absorbed than fructose. In the series with cats more stringent experimental conditions were attained. The animals were pithed in deep ether narcosis, artificial respiration was started, and the application of the anesthetic discontinued. Two or all three sugars were tested in succession on the same intestinal loop and the order of introduction of the sugars was changed in the different animals. The absorption time was 10 minutes. Five experiments are recorded. Galactose was absorbed at about half the rate of that of glucose, fructose at a still smaller rate. In our experiments and in those of Nagano galactose was absorbed at a slightly greater rate than glucose and this was also the case in observations of Hoeber (1899) on dogs and Hédon (1900) on rabbits. It is possible that the results of Hewitt as regards galactose were due to the different species used. On the other hand it is interesting to note that our experiments on rats, with ten times more concentrated sugar solutions, gave the same order in the rate of absorption of the different sugars as those of Nagano on dogs.

These results offer another striking example of the selective permeability of living membranes and in this connection reference

might be made to the work of Hamburger (1922) on the perfused frog kidney. He showed that of the isomeric sugars some are retained by the glomerular membrane,[4] while others are allowed to pass unhindered into the urine. Still more remarkable is the fact that the glomerular membrane is sensitive to such insignificant changes in the sugar molecule as are represented by the $\alpha$ and $\beta$ modifications. From *d*-galactose and *l*-xylose only the $\alpha$ form is retained while the $\beta$ form is completely excreted in the urine. Hamburger on the basis of the well known scheme of Clowes of the transformation of an oil in water into a water in oil emulsion believes that the sieve theory is an adequate explanation for the selective permeability of the glomerular membrane.

Since equal amounts of sugar pass from the intestine into the blood stream in equal periods of time, the absorption is comparable with a continuous intravenous infusion at a constant rate. Woodyatt, Sansum, and Wilder (1915) determined the sugar tolerance of normal rabbits, dogs, and men by the continuous intravenous infusion method. They found that if glucose was infused at a rate of 0.85 gm. per kilo of body weight, per hour, no glycosuria was produced, even if the infusion was extended over many hours. This is a much lower tolerance than was observed on rats during intestinal absorption. These animals after a 48 hour fast absorb 1.78 gm. glucose per kilo per hour over long periods without showing glycosuria. This value would be reduced to 1.54 gm. per kilo per hour if the original body weight is taken as a basis. It could be objected that the rats showed no glycosuria on account of their depleted glycogen stores. This might have increased their capacity to assimilate glucose. However, in control experiments on non-fasting rats the same amount of glucose could be given without causing sugar excretion in the urine. There is also no reason to believe that rats that have not been fasted absorb glucose at a slower rate than fasted animals. It would seem, there-

[4] In the light of recent work on the physiology of the kidney it seems more probable that the selective permeability of the kidney is due to a selective reabsorption in the tubular apparatus rather than to a selective filtration through the glomerular membrane. It seems of interest in this connection that in recent experiments the rate of absorption of different hexoses and pentoses from the peritoneal cavity was found to be identical (Cori and Goltz, 1925, *b*).

fore, that the glucose tolerance when tested by the intestinal route is higher than when tested by the intravenous route. This discrepancy could be explained by assuming that the rat has a higher glucose tolerance than rabbit, dog, or man. Yet Sansum and Woodyatt (1917) in recalculating the data of Fisher and Wishart (1912–13) found that dogs absorb between 1.6 to 1.7 gm. glucose per kilo per hour, a value which approaches closely that found in our experiments on rats. A still greater discrepancy between the tolerance when measured by the intestinal and the intravenous route exists for fructose. The intravenous tolerance limit for this sugar, as determined by Woodyatt, was 0.15 gm. per kilo per hour. The rats absorbed this sugar at a rate of 0.77 gm. per kilo per hour or 0.67 gm. per kilo per hour if the original body weight is taken as basis. Fructose was not excreted in the urine. The tolerance by the intestinal route was therefore four to five times higher than by the intravenous route. Since it could again be objected that the results obtained on different species are not strictly comparable, it is proposed to determine the intravenous tolerance limit for glucose and fructose on rats.[5] There are numerous experiments on record which were taken as evidence that fructose is better tolerated and better utilized than glucose. Fructose has also been given on that assumption to diabetic patients. The literature on this subject has recently been summarized by Isaac (1920). The apparently better utilization of fructose than glucose has been deducted from a comparison of the blood sugar curves and the sugar excretion in the urine following the ingestion of these sugars by the same patient. However, the fact that fructose is absorbed at a much slower rate than glucose has not been taken into consideration in these experiments and the results might equally well be explained on that basis.

Woodyatt found an intravenous tolerance limit for galactose of about 0.1 gm. per kilo per hour. The rats absorbed this sugar at a rate of 1.96 gm. per kilo per hour or 1.69 gm. per kilo per hour, if based on the original body weight. In several experiments close to 50 per cent of the total amount of galactose absorbed was eliminated in the urine. Experiments are in progress to elucidate this observation further.

[5] Since the above has been written the intravenous tolerance of rats was found to be between 2.2 and 2.5 gm. glucose per kilo per hour (Cori, 1925).

## XI.

### SUMMARY.

1. A method for the quantitative study of absorption by the whole intestinal tract has been described. The amount of substance absorbed per unit of body weight per hour of time has been called absorption coefficient.

2. A proportionality between the absorbing surface of the intestine and the body weight has been demonstrated for growing male and female rats.

3. 50 per cent solutions of hexoses (glucose, galactose, fructose, and mannose) are absorbed from the intestine at a rate which is constant for each sugar. Even after the absorption of 70 per cent of the sugar originally introduced the rate of absorption is not diminished. Hence the rate of absorption of hexoses is independent of the absolute amount and also of the concentration of sugar present in the intestine. If the amount of sugar absorbed is plotted against time a straight line is obtained (see Fig. 1).

4. 25, 50, and 80 per cent glucose solutions are absorbed at the same rate, which is considered as additional evidence that the rate of absorption is independent of the sugar concentration present in the intestine.

5. The following order in the rate of absorption has been found: galactose > glucose > fructose > mannose > xylose > arabinose. The selective permeability of the intestinal membrane for stereoisomeric sugars has been emphasized.

6. It has been shown that a hypertonic sugar solution is diluted in the stomach by the excretion of water.

7. The water content of the blood, liver, and muscle is not changed during the absorption of a hypertonic sugar solution, showing that the vital organs are protected against dehydration, whereas the skin shows a marked diminution in the water content.

8. Glucose, when fed to rats in as large amounts as 15 gm. per kilo does not lead to a sugar excretion in the urine. From galactose close to 50 per cent of the total amount absorbed is eliminated in the urine.

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# THE RELATION OF LEG WEAKNESS IN GROWING CHICKS TO MAMMALIAN RICKETS.

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PLATE 2.

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The beneficial effect of known antirachitic agents—cod liver oil and ultra-violet radiation—upon the condition known as "leg weakness" in young chicks, has led to the assumption that this condition is identical with mammalian rickets. To cite only a few of the statements indicating the general belief in this identity, Hughes (1) says that, "The lesions are the same and the conditions under which it is produced are the same as those which cause rickets." Probably the most extensive work in this field has been done by Hart, Steenbock, and their coworkers. In their more recent papers they appear definitely to regard leg weakness as identical with rickets. Thus "The fact that the baby chick is very susceptible to rickets. . . . led us to investigate," etc. (2); "We have successfully prevented and cured rickets in chickens by the administration of cod liver oil" (3); and in a popular article (4) one of the chapters is headed with the caption "Leg Weakness is Rickets." In a paper by one of us (Dunn (5)) the same view-point, based on the identity of the biological reactions, was maintained.

None of the numerous papers which have appeared during the past few years has dealt specifically with the histological changes in the bones. Thus there is at present no morphological evidence for the identity of the two conditions. It was with a view to filling this lack that we undertook a comparison of the bones of a series of young chicks in which leg weakness had been experimentally produced, with those of chicks receiving an adequate supply of the antirachitic factor.

*Material and Methods.*

The chicks were placed upon the experimental diet immediately after hatching. The basal diet used for the production of leg weakness was the same as that employed by Hart, Halpin, and Steenbock (6), consisting of white corn 97 parts, $CaCO_3$ 2 parts, NaCl 1 part, with pasteurized skim milk *ad libitum*.

The conditions under which the chicks were reared were the same as those described by Dunn (5). Pine sawdust was supplied as litter, and direct sunlight excluded.

To supply control material for normal bone structure, chicks from the same families, raised under identical conditions, were given the following complete diet.

| | |
|---|---|
| Yellow corn | 70 |
| Wheat middlings | 20 |
| Beef scraps | 5 |
| $CaCO_3$ | 4 |
| NaCl | 1 |

A supplement of 2 per cent cod liver oil was incorporated in this mixture and skim milk provided *ad libitum*.

A further group was reared from hatching on a diet of yellow corn 97, $CaCO_3$ 2, NaCl 1. The purpose of this ration was to determine what modifications of bone structure might result from the introduction of the vitamin A factor alone.

Two groups, one of 10 and one of 16 chicks, were raised on the white corn ration, with the addition in the first case of approximately 5 per cent of a 1 to 150 dilution in glycerol of the active concentrate of the non-saponifiable fraction of cod liver oil. With the second group, the same preparation was incorporated in the ration to the amount of 2 per cent of a 1 to 75 dilution. This makes a final concentration of 1 part of the concentrate in 3000 parts of white corn ration in the first experiment, and of 1 part in 3700 parts of ration in the second. The proved curative dose of this preparation for rat rickets was 0.1 cc. per day of the 1 to 75 glycerol suspension.[1]

*Histological Methods.*—The bones were fixed in Müller-formol,

[1] We are indebted to Mr. T. F. Zucker for supplying us with this preparation, and to Mrs. Margaret Gutman Newburger for information as to the antirachitic potency of the sample used.

TABLE I.

*Results of Feeding Experiments with Young Chickens.*

All chicks were reared from hatching time on laboratory tables covered with washed sand and pine sawdust. Windows were closed throughout all experiments.

| Experiment No. | No. of chicks. | Basic diet. | Supplement. | No. of chicks with leg weakness. | Age at appearance of leg weakness. |
|---|---|---|---|---|---|
| | | | | | *days* |
| N 10 | 13 | White corn. * | None. | 8† | 16–24 |
| N 15 | 6 | " " * | " | 6 | 19–37 |
| N 13 | 10 | " " * | 5 per cent D.‡ | 5§ | 17–25 |
| N 18 | 16 | " " * | 4 " " " ** | 13‖ | 24–31 |
| N 12 | 17 | Yellow corn.¶ | 2 " " C.L.O.** | None to 2 mos. | |
| N 17 | 15 | " " ¶ | 2 " " C.L.O.** | " " 2 " | |
| N 14 | 11 | " " ¶ | None. | 9†† | 24–25 |
| N 16 | 12 | Normal ration.‡‡ | 2 per cent C.L.O. | None to 3 mos. | |

* The white corn diet consisted of ground white corn, 97, $CaCO_3$, 2, NaCl, 1, with pasteurized skim milk as drink, *ad libitum*.

† Five chicks died from pneumonia or chilling at 1 week of age.

‡ D designates the active antirachitic non-saponifiable fraction of cod liver oil used in a 1:150 suspension in glycerol; thoroughly incorporated in the ration.

§ Three chicks died from undetermined causes at 10 to 13 days of age; two had not developed leg weakness at 1 month of age.

‖ One died at 9 days; two died at 24 days without previous symptoms of leg weakness.

¶ The yellow corn diet consisted of ground yellow corn, 97, $CaCO_3$, 2, NaCl, 1, with pasteurized skim milk as drink, *ad libitum*.

** Freshly opened cod liver oil-Harris, thoroughly incorporated in the ration.

†† Two chicks killed for study before leg weakness appeared.

‡‡ Our normal ration consisted of:

| | |
|---|---|
| Yellow corn | 70 |
| Wheat middlings | 20 |
| Beef scrap | 5 |
| $CaCO_3$ | 4 |
| NaCl | 1 |

supplemented by a scratch feed of 2 parts of cracked corn and 1 part of cracked whole wheat.

partially decalcified in Müller's fluid, and stained with hematoxylin-eosin and with silver nitrate-safranin. The upper extremity of the tibia was taken for routine study.

## EXPERIMENTAL RESULTS.

Since this paper is primarily concerned with the bone changes associated with leg weakness, the summary given in Table I will suffice to show the results of the experiments as regards the onset of leg weakness, and the protective value of the various additions used.

### *Radiographic Examinations.*

Radiographs of normal and leg weakness bones were taken after fixation, but before decalcification. The radiographic findings varied somewhat in individual birds. The appearance which seemed most typical of the leg weakness bones is illustrated in Fig. 1. The head of the tibia is traversed by a dense shadow, corresponding to the zone of calcified cartilage. Below this is a definitely rarefied zone which does not appear in the normal control of the same age, and which, as becomes evident from the histological comparison, is due to the loss of the primary spongiosa. There is no swelling or deformity of the end of the bone, and the cupping, so characteristic of the rachitic metaphysis, is conspicuously lacking.

### *Histological Examination.*

It may be stated at the outset that in none of the 30 chicks (including normal controls) studied were there present lesions which could be regarded as rachitic. Certain very definite and striking alterations, however, were found, the interpretation of which will be discussed later. It is unnecessary to give in detail descriptions of the individual chicks, since with slight variations the appearances were identical in all essentials. The following may be taken to exemplify the series:

*Chick 9764.*—Hatched Jan. 31, 1925. Fed a diet of white corn 97, $CaCO_3$ 2, NaCl 1, skim milk *ad libitum*. Developed typical leg weakness and was killed on the 18th day.

Section through upper extremity of tibia.

The zone of proliferating cartilage and the zone of provisional calcifications are greatly increased in depth, with numerous blunt prolongations

extending downward towards the diaphysis. The columnar arrangement of the cartilage cells is only partially preserved. There are numerous large penetrating vessels, anastomosing with those of the epiphyseal cartilage. These are surrounded by loose fibrous tissue. The epiphyseal cartilage contains no ossification center, and is wholly uncalcified.

Calcification of the cartilage in the zone of provisional calcification is quite uniform and complete in the distal portion.

The primary spongiosa is almost absent; there is a notable lack of osteogenetic tissue about the calcified cartilage cells. Such small fragments of bone trabeculæ as remain are very thin and irregular in their disposition and, for the most part, completely separated from the cartilage. Some of the trabecular remnants are apparently in process of resorption, staining as indistinct pinkish purple clumps. There are fairly numerous multinucleated osteoclasts lying against these resorbing bone spicules. Occasionally they are surrounded by a row of flattened cells, but well formed osteoblasts are conspicuously lacking. There is no osteoid visible.

The cortex of the bone is likewise much rarefied, and especially in the region of the epiphysis, where it is composed of irregular trabeculæ and in places completely interrupted. Differentiated osteoblasts are not to be found, and bone formation is evidently in abeyance. The bone corpuscles are small and angular. An osteoid margin is nowhere to be seen. The periosteum is not thickened.

The marrow is the seat of striking and interesting changes. It is everywhere altered into a myxomatous tissue composed of polyhedral connective tissue cells, with branching processes, embedded in a pale mucoid bluish-staining ground substance. No fat cells are seen. There is great reduction in the blood-forming elements, which are represented only by sparsely distributed islands composed chiefly of eosinophil polymorphonuclears and myelocytes. The blood sinuses are not prominent; some appear collapsed.

The foregoing lesions (Fig. 2) present a striking contrast to those encountered in mammalian rickets, either of spontaneous or experimental origin. The epiphyseal cartilage is increased in width, and the columnar alignment is more or less disturbed, but there is no defect in calcium deposition. As can be seen in radiographs, and in sections stained with silver nitrate, the cartilage is traversed by a broad band of calcium; in rachitic bone, the calcium is characteristically absent in the zone of provisional calcification, and the failure of the cartilage to calcify is a fundamental feature of the rachitic state.

In the subchondral zone, there are equally striking contrasts. In the chicks, there was in every case a marked impoverishment of the spongy trabeculæ. Small fragments, undergoing resorption

and often surrounded by giant cells, marked the site of the original trabeculæ. No new bone was being formed about the cartilage. This complete arrest of endochondral ossification was most striking. In rachitic bone, the subchondral region is occupied by broad, irregular trabeculæ, composed largely of osteoid, and surrounded by distinct and presumably active osteoblasts. Multinucleate cells are rare.

TABLE II.

*Comparative Histological Lesions in Leg Weakness of Chicks, and in Experimental Low Phosphorous Rickets.*

| Tissue. | Leg weakness. Chicks. | Experimental rickets. Rats. |
|---|---|---|
| Cartilage..... | Moderately widened. | Very greatly widened. |
| Calcification of cartilage..... | Present. Occasionally small areas of defective calcification. | Absent. |
| Spongiosa..... | Absent or reduced to small fragments undergoing resorption. | Broad trabeculæ, composed largely of osteoid. |
| Osteoblasts..... | Indistinct or absent. No osteogenesis. | Large, conspicuous. Osteogenesis active. |
| Osteoclasts..... | Numerous. | Rare or absent. |
| Cortex..... | Thin. No osteoid margin. | Calcified portion often thinned, but there are large endosteal and periosteal osteoid thickenings. |
| Osteoid..... | Practically absent. | Great excess. |
| Marrow..... | Fibroid or myxomatous transformation. | Little marrow in metaphyseal region because of osteoid excess. Diaphyseal marrow not much altered. |

An excess of osteoid tissue, perhaps the most significant and distinctive feature of rachitic bone, was never present in the chick preparations. Indeed, the amount of osteoid seemed diminished in comparison with that normally found in the healthy growing control of the same age.

Fibrosis of the marrow in the region of the epiphysis occurs in the more severe forms of human rickets, but is not pronounced

in the experimental rat rickets produced by low phosphorous diets. The majority of chicks with leg weakness showed a striking fibromyxomatous transformation of the marrow, with depletion of the blood-forming cells. This was especially marked in the subchondral area, but in some preparations a strip of fibrous marrow extended along the inner surface of the cortex for a considerable distance.

The periosteum was unaltered, and the marked thickening which is so striking a feature of rachitic bone, did not occur.

In Table II the skeletal changes in the two conditions are compared in tabular form.

The substitution of yellow corn for white not only delayed the onset of the symptoms of leg weakness but produced a definite improvement in the bone structure. This is illustrated in the following protocol.

*Chick 9825.*—Fed on diet of yellow corn 97, $CaCO_3$ 2, NaCl 1. Killed on 21st day, having shown no symptoms of leg weakness.

The zone of provisional calcification in the upper extremity of the tibia is broad and deep, and well calcified in its distal portion. The spongiosa is composed of numerous well calcified narrow trabeculæ, with a distinct, but not broad, osteoid margin, and conspicuous cuboidal osteoblasts. The cellular marrow extends to the cartilage, and the myxomatous change seen on the white corn diet is absent. The cortex is relatively stout. The central cartilage of the diaphysis is completely resorbed.

There is practically no difference therefore at this age from the normal bone structure exhibited by birds on a complete diet. It would seem that the administration of the vitamin A as contained in the yellow corn has brought about more active osteogenesis in the young growing bone.

As shown in Table I, the administration of an actively antirachitic substance in the form of a concentrate of the non-saponifiable fraction of cod liver oil, was not efficacious in preventing the onset of leg weakness in chickens on a white corn diet. Thus of ten chicks in this group, four died of other causes before the age of incidence of leg weakness, but the remaining six acquired the typical symptoms at the usual time. The following protocol brings out the fact that addition of this active antirachitic substance failed entirely to bring about an improvement in the histological bone structure.

*Chick 9860.*—Placed on white corn ration Mar. 30, with the addition of 5 per cent of a 1:150 dilution of the active concentrate. Symptoms appeared on the 15th day and the chick died with typical leg weakness on the 21st day.

Section through upper extremity of tibia.

There is increased depth and irregularity in the growing cartilage, accompanied by massive, though unevenly distributed, deposition of calcium. There is little evidence of active bone growth at the epiphyseal junction. The blunt calcified cartilage processes lie exposed and no bone trabeculæ are being formed about them. There is no penetration of blood vessels, nor erosion of the cartilage cells. The marrow in the vicinity of the cartilage and further down the shaft is much depleted of blood-forming elements, and shows the same fibromyxomatous structure as in Chick 9764, and other birds on the white corn diet without additions. The cortex is well calcified without visible osteoid margin. Osteoblasts are indistinct.

The picture presented is thus virtually the same as that on the white corn ration without antirachitic substance, and one may conclude that the addition of an active antirachitic agent alone, as contrasted with the addition of whole cod liver oil, brings about no improvement in the bone structure. There is, in other words, no stimulating effect upon the osteogenetic function of the marrow.

## DISCUSSION.

From the anatomical point of view, it is plain that the bone lesions found in the leg weakness of young growing chicks, at least with the experimental diets used, are not those of rickets. The essential feature of the rachitic state is the *failure of calcium deposition*, both in the growing cartilage and in the new formed osteoid tissue. In the bones of leg weakness chicks, with the diets used, the central change seems rather to be a *failure of osteogenesis*, with continuing and possibly exaggerated resorption of previously formed bone. Were it not for the absence of hemorrhage, the lesions would far more closely resemble the effects of scurvy than of rickets. Indeed, the resemblance is sufficiently striking to bring up anew the question as to whether the absence of antiscorbutic vitamin may not be a factor in determining the character of the lesions. The literature contains a number of positive statements bearing upon this point. Thus Hart, Halpin, and Steenbock (7) state that the administration of orange juice has no beneficial effect in the prevention or cure of leg weakness. Emmett

and Peacock (8) found no growth stimulation upon the addition of tomato juice, and conclude that vitamin C is not essential. Mitchell, Kendall, and Card (9) have made similar observations. Plimmer and Rosedale (10), Sugiura and Benedict (11), Shorten and Ray (12), and Carrick and Hauge (13), are all agreed that chickens do not require the antiscorbutic vitamin, and the last authors have shown that, as in the case of the rat, the antiscorbutic substance is contained in abundance in the liver even when the animal is maintained for a long period on a vitamin C-deficient diet.

It is thus generally held that birds are insensitive to lack of antiscorbutic vitamin, and possibly have the power of synthesizing it. So far as we are aware, detailed histological studies of the bones of chicks on diets deficient only in vitamin C have not been published. We have, however, examined sections of bones of chicks receiving the preventive addition of cod liver oil, and since this agent which does not contain the antiscorbutic factor effectually overcomes the failure of osteogenesis and the accompanying marrow fibrosis, there can be little reason for regarding these changes as scorbutic.

We are thus dealing with a condition which is pathologically distinct from rickets, but which in its biological reactions shows undoubtedly close relationship to this disease. However, the biological resemblance, though intimate, is not complete. Thus the concentrate of the non-saponifiable fraction of cod liver oil (Zucker), which is actively antirachitic in rats, failed to protect against leg weakness (Table I).

It might be thought that the bone changes in leg weakness are merely the effect of failure of growth. Fasting, and indeed faulty nutrition from any cause in young animals, is accompanied by arrest of bone formation. In a number of the chicks examined growth during the experimental period up to the time that symptoms developed as judged from the weight curves, was virtually normal.[2] The lesions, therefore, cannot be attributed to inanition, nor can it be maintained that the rachitic lesions are masked by the malnutrition. While it is in general true that the severity of

[2] Unpublished data indicate that the growth of chicks which develop leg weakness does not differ significantly from the growth of chicks which do not develop leg weakness, until the onset of this condition.

rachitic lesions is greatest in animals showing rapid growth and that fasting tends to induce healing, most intense rachitic lesions may and frequently do develop in experimental animals though the weight curve remains stationary.

Because of the addition of skim milk to the basal ration, the phosphorus content of the chick diets is considerably higher than in the low phosphorus diets used for the experimental production of rickets in rats. We have calculated that the average daily phosphorus intake of chicks on the white corn-skim milk ration is about 33 mg. during the first 3 weeks. On the Sherman-Pappenheimer low phosphorus rickets-producing diet, the average daily intake of phosphorus is probably 12 mg. or less. While these figures have no claim to accuracy, and do not take into account the availability of different forms of phosphorus, or actual absorption, they do indicate that the leg weakness ration is relatively higher in phosphorus than one of the standard rickets-producing diets.

We have tested the same diet which produced leg weakness in chicks (namely, white corn 97, $CaCO_3$ 2, NaCl 1, skim milk *ad libitum*) upon a small series of rats. Direct sunlight was excluded. Growth from the 3rd to the 7th week was normal and histological examination of the ribs in three rats showed no rachitic changes. The skim milk was then withdrawn from the diet, and the three remaining rats examined after a further period of 3 weeks. All showed grossly and microscopically pronounced and typical rachitic lesions. Thus it is seen that leg weakness diet is antirachitic with milk supplement; rachitic without milk.

It is quite possible that under the conditions of the experiments, the phosphorus assimilation was sufficient to cover the calcification of the inactively growing skeleton, though not adequate for optimal bone growth. A similar condition obtains when to Diet 84 (Sherman and Pappenheimer (14)) are added 75 mg. per cent of phosphorus, which amount suffices to protect against the development of rachitic lesions, but is undoubtedly below the amount necessary for normal bone growth. Whether it will be possible by further reduction of the phosphorus intake to produce true rachitic lesions in the bones of young chicks remains to be demonstrated.

The investigations of Hess (15) and of Casparis, Shipley, and Kramer (16) have shown that egg yolk possesses antirachitic

properties comparable to those of cod liver oil. Bethke and Kennard (17) were able to raise chicks indoors to maturity by the addition of 15 per cent egg yolk in place of cod liver oil, and Collier also found that young chicks may be successfully reared without exposure to direct sunlight or administration of cod live oil when hard boiled eggs are added to the ration. The Wisconsin Experiment Station (4) has also reported that a ration of white corn, middlings, and skim milk may be made adequate for normal growth by the addition of one egg per day to the ration for 30 chicks.

It seems established, therefore, that egg yolk is not only antirachitic, but also protects against leg weakness. The question thus arises whether the absence of rachitic lesions in chicks may not be due to the storage of yolk or its active component in the tissues during the experimental period. That there is no permanent storage of the substance which prevents leg weakness is shown by the fact that this condition frequently appears in the 3rd week, and occasionally as early as the 17th day. This, however, gives no evidence as to the possible storage of an antirachitic substance, and the question must be left open until typical rickets has been experimentally produced in chicks.

## CONCLUSIONS.

1. Radiographic and histologic study of a series of bones of young growing chicks with experimentally produced leg weakness showed no rachitic changes.

2. The bone lesions found were arrest of osteogenesis, osteoporosis, and fibromyxomatous transformation of the marrow.

3. Administration of the active antirachitic concentrate of the non-saponifiable fraction of cod liver oil did not prevent the onset of leg weakness, nor modify the character of the bone lesions.

4. The addition of whole cod liver oil prevented leg weakness, and brought about normal bone structure.

5. The diet which produced typical leg weakness in chickens was fed to young rats for 4 weeks but did not produce rachitic changes in their bones.

*Addendum.*—While this manuscript was in press there appeared an article by Hart, Steenbock, and Lepkovsky (19) in which the use of a white

corn ration for testing the antirachitic potency of stored cod liver oil is criticized on the ground that the results are complicated by vitamin A deficiency. Experiments are reported in which the basal ration is composed of yellow corn 97, calcium carbonate 2, and sodium chloride 1, with skin milk *ad libitum*. This diet is said to produce a distinctly rachitic condition, recognized by the shambling gait, ruffled feathers, low ash content of the alcohol-extracted bones, and microscopically, by the wide uncalcified area of proliferating cartilage.

The microscopic examination was carried out on bones decalcified for 7 to 10 days in 10 per cent nitric acid.

We should like to make the following criticism. (1) A diminished ash content in itself does not establish rickets, but is found in simple osteoporosis; (2) acid decalcification makes the microscopic recognition of calcium unreliable; (3) Fig. 1 showing wide rachitic proliferating cartilage is made from a section which does not pass longitudinally through the shaft of the bone, and the widening of the cartilage shown in the picture may be due to the fact that the joint cartilage has been cut tangentially; (4) excess of osteoid, the most characteristic histological feature of rachitic bone, is not mentioned, nor does Fig. 1 suggest its presence.

It does not seem to the writers that the diagnosis of rickets is warranted from the data presented. In our experience (see protocol of Chick 9823) a yellow corn diet identical with that used by Hart, Steenbock, and Lepkovsky did not produce rickets.

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EXPLANATION OF PLATE 2.

FIG. 1. Radiographs of bones of normal and leg weakness chickens.

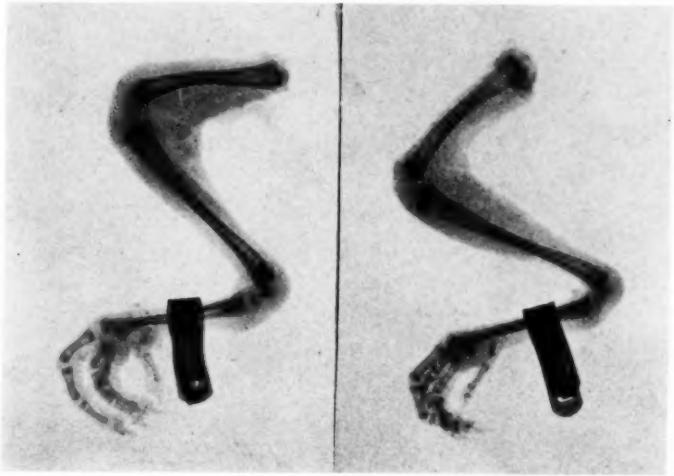
*a*. Chick 9799, killed at 15 days, normal.

*b*. Chick 9860, died with leg weakness at 21 days.

FIG. 2. Photomicrographs of bones of normal and leg weakness chicks (stained with silver nitrate-safranin).

*a*. Chick 9772, normal, killed at 15 days. Normal tibia.

*b*. Chick 9768, died with leg weakness at 24 days. Tibia showing normal calcification of cartilage, absence of spongiosa, rarefaction of cortex.



a b

FIG. 1.

a b

FIG. 2.

(Pappenheimer and Dunn: Leg weakness in chicks.)

# THE INFLUENCE OF INSULIN, ADMINISTERED ORALLY AND SUBCUTANEOUSLY, IN PHLORHIZIN DIABETES.

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Absorption of insulin from the alimentary tract of the depancreatized dog is easily demonstrated (1, 2, 3). But the depancreatized dog has no trypsin; hence is not a suitable animal for study of the problem of oral administration of the antidiabetic hormone. Impressed by Ringer's (4) experiments showing evidence of combustion of sugar after subcutaneous administration of insulin in the phlorhizinized dog, we turned to this type of subject as more representative of the alimentary status of the severely diabetic human subject. The orientation experiments were not very successful in showing absorption when enteric coated tablets similar to those which had been employed with depancreatized dogs were used. Several other types of tablet also were without effect and insulin given in oleic acid or in aqueous solution with sodium oleate, was likewise ineffective. In emulsions of neutral oils, as suggested by Salén (5), it had but a feeble effect, if any, on sugar excretion although the blood sugar was increased. One type of tablet has given mildly positive results. Its effect in contrast with the effect of insulin given subcutaneously is seen in Charts 1, 2, and 5. All of the insulin used was prepared in this laboratory.

## EXPERIMENTAL PART.

### *Orientation Experiments.*

*Dog 1.*—A black and white long haired, female mongrel weighing 11.0 kilos received 200 gm. lean meat daily. 1 gm. phlorhizin in olive oil administered daily at 5.00 p.m. beginning on Mar. 31. On experiment days feeding was postponed until after last catheterization. Urine collection

was begun on Apr. 2. Periods in this and all other experiments always terminated by catheter.

The day's urine was divided into short periods for the purpose of exhibiting the fluctuations in excretion which would necessarily enter into the evaluation of the results of oral administration if one were to determine just when a tablet began to be effective. Tablet 7, used in this and the next experiment contained approximately 1 Rochester unit (6) of insulin in the form of our salt precipitate (7) combined with sodium oleate. It was

TABLE I.

*Dog 1.*

| Date and hour. | Total D. | Total N. | D:N | D per hr. | N per hr. | Remarks. |
|---|---|---|---|---|---|---|
| 1924 | gm. | gm. | | gm. | gm. | |
| Apr. 3, 9.00 a.m. | 19.80 | 5.184 | 3.82 | 1.16 | 0.305 | |
| 12.00 m. | 5.64 | 1.425 | 3.92 | 1.88 | 0.475 | |
| 3.00 p.m. | 4.70 | 1.368 | 3.43 | 1.56 | 0.456 | |
| 5.00 " | 3.10 | 0.950 | 3.26 | 1.55 | 0.475 | |
| Total for 23 hrs. | 33.24 | 8.927 | 3.72 | 1.44 | 0.388 | Average hourly for 23 hrs. |
| Apr. 4, 8.00 a.m. | 16.29 | 6.148 | 2.65 | 1.09 | 0.409 | |
| 10.00 " | 3.28 | 0.978 | 3.37 | 1.64 | 0.486 | Four No. 7 tablets, 10.05 a.m. |
| 12.00 m. | 2.75 | 0.803 | 3.41 | 1.37 | 0.401 | Four No. 7 tablets, 12.05 p.m. |
| 2.00 p.m. | 2.91 | 1.063 | 2.73 | 1.45 | 0.531 | Four No. 7 tablets, 2.05 p.m. |
| 4.00 " | 2.44 | 0.860 | 2.83 | 1.22 | 0.430 | |
| 6.00 " | 2.52 | 1.005 | 2.51 | 1.26 | 0.502 | |
| Total for 25 hrs. | 30.19 | 10.857 | 2.78 | 1.21 | 0.434 | Average hourly for 25 hrs. |
| Apr. 5, 8.00 a.m. | 19.53 | 7.890 | 2.47 | 1.39 | 0.563 | |

enteric coated with a preparation of salol. The average D:N ratio for Apr. 3 shows that the animal was thoroughly phlorhizinized but there was a gradual decline of the D:N in the afternoon hours (Table 1). This is the result of a gradual falling off in the excretion of sugar with no change, after 12 noon, in the excretion of nitrogen. The D:N was low in the night urine which terminated at 8.00 a.m., Apr. 4, due to a decline in the hourly excretion of sugar below the average level for the day before and a somewhat increased excretion of nitrogen above the average level of the day before.

Starting at 10.05 three doses of four of the No. 7 tablets each were given

at 2 hour intervals. The D:N ratio having risen from 2.65 to 3.37 at 10.00 a.m. rose slightly farther the first 2 hours, then fell off to 2.73, and finally to 2.51 at 6.00 p.m. Without the controls of the previous day it was would have been easy to conclude from this result that the insulin tablet had been notably successful, but the decline is almost parallel with that of the day before when there was no treatment. Moreover, while the sugar excretion had fallen from 1.64 gm. per hour at 10.00 a.m. to 1.26 at 6.00 p.m., the general level of the nitrogen excretion had risen. Next morning while the excretion of sugar in the night urine had risen slightly, the excretion of nitrogen had risen considerably, resulting in a lower D:N ratio. The only encouragement for mouth administration we could derive from this experiment was in the diminished total excretion of sugar from 33.24 gm. for 23 hours to 30.19 gm. for 25 hours. The larger excretion of sugar, by over 3 gm., for the 14 hours terminating on the morning of the 5th, than for the 15 hours terminating on the morning of the 4th also indicates that some sugar had been retained under the influence of the insulin. One must not hastily conclude that this was mere renal effect; for if the sugar were deposited as glycogen during the hours when the insulin was effective, it would be released again as sugar under the influence of the phlorhizin as soon as insulin wore off.

One other experiment with the same tablet was performed on this dog on the 8th. There was a similar though smaller decline in the hourly excretion of sugar in the 4 hours following last administration of the tablet with no accompanying rise in nitrogen. But the next morning a much higher excretion of nitrogen signalized the well known nitrogen lag. The dog had been so long under phlorhizin that no adequate control period following the administration could be obtained.

*Dog 2.*—A similar experiment with Tablet 7 was carried out on Dog 2, which had been fasting for 7 days and under the influence of phlorhizin for 5 days. This time there was no falling off in the hourly dextrose elimination and no material change in the hourly excretion of nitrogen up to 6 hours. The experiment however was lacking in adequate control periods. No further trials were made with this identical tablet, but with Dog 5 (see p. 741) one experiment was performed in which a pill of similar composition was used and another in which a more highly purified insulin was administered with sodium oleate in solution.

We next turned our attention to the administration of insulin in suspensions or emulsions of fatty acids or neutral oils after the manner of Salén who claims favorable effects upon human subjects from this procedure. The first experiment which was performed on Dog 2 failed because the urine was contaminated by diarrhea.

*Dog 3.*—A brown and white, long haired, female mongrel weighing 10 kilos was given 1 gm. phlorhizin in olive oil at 4.00 p.m. daily beginning Apr. 22 and continuing until Apr. 26. The effect of the phlorhizin not being satisfactory by this mode of administration, that is, the D:N ratio remaining high, resort was had on the 26th to the older method of solution of the phlorhizin in 1 per cent sodium carbonate and two doses of 1 gm. each were

given daily. This was kept up for 2 days after which the oil method was resumed. The ratio came down to a satisfactory level on the 28th when the experiment shown in Table II was started.

The after control period was ruined by the occurrence of severe diarrhea and consequent contamination of the cage urine. Possibly for this reason the experiment does not give any clear evidence of insulin absorption. The D:N ratio for the 6 hours just preceding the administration was 4.34 and for the 6 hours immediately following 3.81 but the effect is due mainly to a considerable increase in the rate of nitrogen excretion as compared with the rate of sugar excretion. Quite possibly the protein material contained in the insulin preparation (it was a crude form of insulin) contained extractive nitrogen which contributed to the result. As a rule, of course,

TABLE II.

*Dog 3.*

| Date and hour. | Total D. | Total N. | D:N | D per hr. | N per hr. | Blood sugar. | Remarks. |
|---|---|---|---|---|---|---|---|
| *1924* | *gm.* | *gm.* | | *gm.* | *gm.* | *mg.* | |
| Apr. 28, 3.50 p.m. | 11.11 | 3.236 | 3.43 | 1.51 | 0.441 | | |
| Apr. 29, 9.15 a.m. | 29.26 | 7.266 | 4.09 | 1.71 | 0.417 | | 30 Rochester |
| | | | | | | 2.00 p.m. | units in- |
| 2.15 p.m. | 6.57 | 1.512 | 4.34 | 1.31 | 0.302 | 45 | sulin in 50 |
| | | | | | | 3.45 p.m. | cc. oleic |
| 5.10 " | 4.71 | 1.151 | 4.09 | 1.61 | 0.394 | 50 | acid at 2.05 |
| | | | | | | 5.10 p.m. | p.m. |
| 8.20 " | 3.77 | 1.074 | 3.51 | 1.22 | 0.348 | 48 | |
| | | | | | | 8.20 p.m. | |
| | | | | | | 50 | |
| Total for 6 hrs. | 8.48 | 2.225 | 3.81 | 1.41 | 0.371 | | |

when any form of protein is fed to a diabetic dog, sugar from the protein is excreted before the urea (8). The only justification for reporting the experiment lies in the two facts; namely, that the sugar excretion was smaller after insulin than it should have been, judging by the nitrogen, and that there was a measurable rise in blood sugar. If a small amount of insulin had been absorbed and if insulin acted to antagonize phlorhizin as regards its effect upon the kidney these results would be expected. The blood sugars were determined by the Folin-Wu method.

*Dog 4.*—A brown, shaggy collie weighing 8.2 kilos. The animal fasted throughout. A new lot of phlorhizin was used and was given in doses of 1 gm. in olive oil daily beginning Apr. 30. Table III exhibits the results of two attempts to influence the diabetic state by administration by stomach tube of insulin in cod liver oil emulsion. The insulin from a very potent product was dissolved in 10 cc. of distilled water, which was then mixed

with 20 cc. of cod liver oil and shaken until well emulsified. The first dose was given on May 3, when the D:N ratio was at a satisfactory level, showing complete phlorhizination. There was no effect until 6 hours had elapsed at which time the D:N ratio was distinctly lower, due to a diminished rate of sugar excretion, without change in the nitrogen. The blood sugar also was distinctly raised.

On May 4 two doses of insulin prepared in the same way were given, one at 12.45 p.m. and the other, twice the amount of the first, at 4.05 p.m. Again there was a decline in the D:N ratio from 3.44 at 12.40 p.m. to 3.04 at 10.10 p.m. The blood sugar, determined on this day by the Hagedorn-Jensen method, rose from 56 to 70 mg. per 100 cc. The latter result is probably significant, but it is doubtful whether the former can be so regarded on account of the gradual decline in total metabolism with continuous fasting. The hourly excretion of sugar for the 21¾ hours from 10.55 on the 2nd to 8.40 a.m. on the 3rd is 1.06 gm. For the next 26 hours including the first insulin period it is 0.97 gm., and for the next 23 hours, including the two insulin feedings, 0.96 gm. The average for the following control day is 0.88 gm. There remains the possibility that there was a very slight improvement as indicated by the diminishing hourly excretion from the time of administration of insulin to the late evening hour, with no or very slight change in the nitrogen, which is not seen in the subsequent control day, and indicated also by the rise in blood sugar. Incidentally the higher blood sugar persists to the afternoon of the after control day (see Table III). We regard all of the tests of oral administration of insulin in oil or oleic acid as of doubtful value for they certainly are not significant enough to give encouragement for this form of oral administration to the human patient. The slight changes mentioned may be nothing more than the diminishing effect of the phlorhizin, given in the morning, as the day wears on.

We next turned to a different mode of administration of insulin—one which had proved efficacious in the depancreatized dog (1, 2) namely in solution in dilute sodium hydroxide. As an additional criterion of change we resorted again to the respiratory quotient which had been employed in the study of the depancreatized dog. The closed circuit apparatus, consisting of a chamber ventilated by means of a Benedict Universal Machine was used. Numerous alcohol checks were run but residual analyses of the air were not made in this particular experiment. With this mode of operation the results on the R.Q. are not reliable if the animal moves about or if the dog should urinate in the chamber. No trouble was experienced from either cause with this subject, although there was one questionable quotient on the 2nd day of experiment as appears in Table III. It was probably due to a slight leak.

The D:N ratio on the morning of the 6th was low, 2.67, but rose during the time when the control R.Q.'s were being taken to 3.43. The quotients show clearly that the animal was wholly unable to oxidize sugar. When 60 Rochester units of a potent and carefully assayed preparation of insulin were given in N/20 NaOH at 4.40 p.m. and the animal returned to the

TABLE III.
*Dog 4.*

| Date. | Total D. | Total N. | D:N | D per hr. | N per hr. | Blood sugar. | R.Q. | Remarks. |
|---|---|---|---|---|---|---|---|---|
| *1924* | *gm.* | *gm.* | | *gm.* | *gm.* | *mg.* | | |
| May 2, 10.55 a.m. | 15.10 | 3.360 | 4.49 | | | 35* | | 3rd phlorhizin day. |
| 2.20 p.m. | 4.23 | 0.976 | 4.33 | 1.24 | 0.286 | 36* | | |
| 5.00 " | 3.26 | 0.752 | 4.33 | 1.22 | 0.282 | 33* | | |
| May 3, 8.40 a.m. | 15.57 | 4.158 | 3.74 | 0.99 | 0.266 | | | Phlorhizin at 8.30 a.m. |
| 9.45 " | | | | | | 35* | | |
| 2.00 p.m. | 5.49 | 1.449 | 3.78 | 1.03 | 0.272 | 36* | | 30 Rochester units E-29 in cod liver oil at 2.10 p.m. |
| 5.15 " | 3.52 | 0.941 | 3.74 | 1.08 | 0.289 | 61* | | |
| 8.15 " | 2.71 | 0.829 | 3.26 | 0.90 | 0.276 | 54* | | |
| May 4, 10.35 a.m. | 13.44 | 4.301 | 3.12 | 0.93 | 0.300 | | | Phlorhizin at 9.30 a.m. |
| 12.40 p.m. | 2.08 | 0.604 | 3.44 | 1.00 | 0.290 | 56† | | 30 Rochester units E-29 in cod liver oil emulsion at 12.45 p.m. |
| 4.00 p.m. | 3.54 | 1.139 | 3.10 | 1.06 | 0.342 | 54† | | 60 Rochester units E-29 in cod liver oil emulsion at 4.05 p.m. |
| 7.00 " | 3.08 | 0.897 | 3.43 | 1.03 | 0.299 | 61† | | |
| 10.10 " | 2.96 | 0.974 | 3.04 | 0.94 | 0.308 | 70† | | |
| May 5, 9.40 a.m. | 10.40 | 3.132 | 3.32 | 0.90 | 0.272 | | | Phlorhizin at 9.20 a.m. Control day. |
| 1.40 p.m. | 3.57 | 1.062 | 3.36 | 0.89 | 0.265 | 85† | | |
| 6.20 " | 4.02 | 1.283 | 3.13 | 0.86 | 0.275 | | | |
| 10.10 " | 3.49 | 1.037 | 3.36 | 0.91 | 0.271 | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| May 6, 9.45 a.m. | 10.15 | 3.160 | 3.21 | 0.88 | 0.273 | | | Phlorhizin at 9.30 a.m. |
| 1.10 p.m. | 2.70 | 1.009 | 2.67 | 0.79 | 0.295 | | | |
| 2.33–3.22 " | | | | | | | 0.673 | |
| 3.22–4.12 " | | | | | | | 0.681 | |
| 4.35 " | 2.85 | 0.831 | 3.43 | 0.84 | 0.244 | | | 60 Rochester units E-29 with 80 cc. N/20 NaOH at 4.40 p.m. |
| 6.26–7.40 " | | | | | | | 0.718 | |
| 7.40–9.01 " | | | | | | | 0.670 | |
| 9.01–9.50 " | | | | | | | 0.676 | |
| 10.20 " | 5.18 | 1.629 | 3.18 | 0.90 | 0.283 | | | |
| May 7, 10.10 a.m. | 9.80 | 3.104 | 3.15 | 0.83 | 0 262 | | | Phlorhizin at 9.30 a.m. 60 Rochester units E-29 with 80 cc. N/20 NaOH plus 20 gm. glucose at 1.45 p.m. |
| 1.35 p.m. | 2.21 | 0.708 | 3.12 | Incomplete. | | | | |
| 3.19–4.09 " | | | | | | | 0.685 | |
| 4.09–5.00 " | | | | | | | 0.623(?) | |
| 5.00–5.57 " | | | | | | | 0.723 | |
| 6.10 " | 0.96 | 0.077 | 12.46 | Incomplete. | | | | |
| May 8, 9.55 a.m. | 19.00 | 3.913 | 4.85 | 1.23 | 0.252 | | | |

* Bloods by Folin-Wu method.
† Hagedorn-Jensen.

TABLE IV.
*Dog 5.*

| Date. | Total D. | Total N. | D:N | D per hr. | N per hr. | Remarks. |
|---|---|---|---|---|---|---|
| *1924* | *gm.* | *gm.* | | *gm.* | *gm.* | |
| May 19, 9.45 a.m.–2.30 p.m. | 16.12 | 3.640 | 4.43 | 3.39 | 0.766 | |
| 6.00 p.m. | 10.68 | 2.667 | 4.00 | 3.05 | 0.718 | |
| 10.30 " | 13.51 | 3.234 | 4.17 | 3.00 | 0.718 | |
| May 20, 10.00 a.m. | 30.48 | 8.092 | 3.76 | 2.65 | 0.703 | |
| 4.15 p.m. | 17.00 | 4.501 | 3.77 | 2.72 | 0.720 | 30 Rochester units E-29 plus 5 gm. Na oleate in 80 cc. water at 4.20 p.m. |
| 9.10 " | 13.88 | 3.640 | 3.81 | 2.82 | 0.741 | |
| 11.45 " | 6.71 | 1.708 | 3.92 | 2.60 | 0.622 | |
| May 21, 10.30 a.m. | 26.04 | 6.664 | 3.91 | 2.42 | 0.619 | 12 pills No. 6 at 10.35 a.m. |
| 2.45 p.m. | 10.87 | 2.884 | 3.76 | 2.55 | 0.678 | |
| 5.45 " | 7.46 | 2.030 | 3.67 | 2.49 | 0.676 | |
| May 22, 9.35 a.m. | 37.31 | 10.248 | 3.64 | 2.35 | 0.647 | 10 tablets (No. 1), 1 every half hour 9.40 a.m. to 2.10 p.m. |
| 4.55 p.m. | 20.49 | 4.564 | 4.49 | 2.80 | 0.622 | |
| 10.15 " | 11.36 | 2.828 | 4.01 | 2.13 | 0.530 | |
| May 23, 9.30 a.m. | 21.74 | 5.488 | 3.96 | 1.93 | 0.487 | Large abscess opened. |
| 1.50 p.m. | 10.20 | 2.604 | 3.91 | 2.35 | 0.601 | |
| 6.15 " | 13.22 | 2.968 | 4.45 | 3.00 | 0.673 | Contaminated by abscess drainage. |

| | | | | | | |
|---|---|---|---|---|---|---|
| May 24, 9.45 a.m. | 3.73 | 1.015 | 3.67 | | | Incomplete, catheter specimen. |
| 12.25 p.m. | 6.41 | 1.869 | 3.43 | 2.41 | 0.702 | 30 Rochester units E-29 plus 15 gm. cod liver oil soap at 12.30 p.m. |
| 3.30 " | 8.26 | 2.464 | 3.35 | 2.68 | 0.800 | |
| 6.50 " | 9.38 | 2.681 | 3.49 | 2.81 | 0.805 | |
| May 25, 10.10 a.m. | 30.67 | 9.268 | 3.30 | 2.00 | 0.604 | |
| 1.10 p.m. | 5.05 | 1.302 | 3.87 | 1.68 | 0.434 | 12 tablets (No. 5), 1 every 20 min., 1.10 to 4.50 p.m. |
| 5.10 " | 7.30 | 1.778 | 4.10 | 1.82 | 0.444 | |
| 11.10 " | 10.00 | 2.422 | 4.12 | 1.66 | 0.404 | |
| May 26, 8.30 a.m. | 16.04 | 3.33 | 4.81 | 1.72 | 0.356 | Dog in good condition up to this point. |
| May 29 | 18.31 | 4.732 | 3.86 | 1.02 | 0.263 | Approximately 18 hr. urine. Dog in coma. |

chamber at 6.00 there was no improvement in the R.Q. up to 9.50 p.m. except a very slight rise in the 1st hour. In the light of subsequent experiences this may be a genuine rise but is insignificantly small compared with what often had been obtained with the depancreatized dog which, of course, has no trypsin to inactive the insulin. There was also no change in the rate of sugar excretion.

Again on the 7th insulin in the weak alkali was given by stomach tube and 20 gm. of Merck's dextrose with it. There was no material effect on the respiratory quotient up to 4 hours from the time of administration.

*Definitive Experiments.*

Having determined upon the time intervals best suited to exhibit the effects of oral administration in comparison with subcutaneous administration, the following experiments were performed in a nearly uniform manner.

*Dog 5.*—Female hound weighing 20.7 kilos. Phlorhizin in oil, 2 gm. daily, beginning May 16. Fasting throughout the experiment. The dog remained in good condition for the entire week of fasting and phlorhizin diabetes, dying finally in coma 3 days after the termination of the experiment.

In this experiment trial was made of the influence of the sodium soap of an unsaturated fatty acid as an antitryptic agent, also of an enteric coated pill containing such a soap and of two different tablets containing malic acid. On May 20 the D:N ratio having fallen to the desired level (Table IV) 30 rabbit units of insulin plus 5 gm. sodium oleate in 80 cc. of water were given by stomach tube at 4.20 p.m. At 9.10 there was a measurable increase in the rate of excretion of both sugar and nitrogen, followed 2 hours later by a decrease in both, the D:N ratio remaining practically stationary. A similar mixture placed directly in the intestine of a dog under ether anesthesia serves often to reduce the ether hyperglycemia very sharply (9). In the present instance, however, the insulin apparently was inactivated by the alkali set free by the soap before it could be absorbed (Table IV).

On the 21st twelve pills containing approximately a Rochester unit each of insulin in the form of a dry powder together with twice its weight of sodium oleate, the whole pressed into a round tablet and coated with a salol preparation, were given at 10.35 a.m. There was no material effect. It has been found sub-

sequently that the enteric coating used with this pill was too resistant to the solvent action of the intestinal juices.

Tablet 1 used on May 22 produced in the second urine period a measurable decrease, both on the sugar excretion and especially on the nitrogen excretion (with consequent rise in the D:N) and this effect persisted over to the next morning. This is one of the most successful tablets we have had. It contained malic acid and has given positive results with human diabetics (10) as well as with diabetic dogs and in ether hyperglycemia. Unfortunately this dog developed a large abscess the same day, which had to be drained next day, but there is no reason to believe the result is vitiated thereby, except when the urine was contaminated directly by the drainage or when the dog lapped up the discharge, which could not occur until after the abscess was opened.

On the 24th a larger amount of soap was used—this time the potassium soap of cod liver oil. It was prepared in the laboratory 2 days before being used and had dried to the consistency of ordinary soft commercial soap. There was a sharp increase in the protein metabolism as witnessed by the heightened output of nitrogen and of sugar, but the D:N ratio was unaffected. It is unlikely that the soap contained a sufficient impurity of protein (4 per cent) to produce such an effect; hence it may be regarded as a genuine increase in the intensity of protein catabolism. Similar, though much milder effects were noted with the smaller dose of sodium oleate and after use of the pills (see Table IV).

Tablet 5 used on the 25th was similar as regards internal composition to Tablet 1. The effect here is somewhat obscured by the extremely low level of metabolism compared with the original level, brought on by the simple process of fasting. We believe there was a genuine reduction in the metabolism attributable to the tablet, but this belief rests mainly upon the results obtained with the next two dogs.

*Dog 6.*—Female Irish terrior. Gave birth to a litter of pups about 4 weeks previously. Pups had been taken away 1 week before phlorhizin was begun. Weight 13 kilos. Dosage of phlorhizin 1 gm. in olive oil daily, beginning May 27, 8 a.m. Fasting throughout the experiment. Urine collection started on 4th phlorhizin day. (Table V.)

TABLE V.
*Dog 6.*

| Date. | Total D. | Total N. | D:N | D per hr. | N per hr. | Blood sugar. | Remarks. |
|---|---|---|---|---|---|---|---|
| *1924* | *gm.* | *gm.* | | *gm.* | *gm.* | *mg.* | |
| May 30, 10.20 a.m.–2.20 p.m. | 8.93 | 2.121 | 4.21 | 2.23 | 0.530 | | 4th phlorhizin day. |
| 8.10 p.m. | 12.05 | 3.073 | 3.92 | 2.06 | 0.527 | | |
| May 31, 10.00 a.m. | 26.04 | 6.636 | 3.92 | 1.88 | 0.497 | | |
| 12.00 m. | 4.39 | 1.024 | 4.29 | 2.19 | 0.512 | 42 | 6 Rochester units P-29 subcutaneously at 12.30 p.m. |
| 2.30 p.m. | | | | | | 38 | 1 hr.'s urine lost. |
| 4.00 " | 3.85 | 0.952 | 4.04 | 1.28 | 0.317 | | D and N per hr. calculated for 3 hrs. 20 gm. Merck's pure dextrose by stomach tube at 4.30 p.m. |
| 4.30 " | | | | | | 36 | |
| 11.50 " | 25.15 | 2.422 | 10.38 | 3.21 | 0.309 | | |
| June 1, 7.50 a.m. | 12.81 | 3.262 | 3.92 | 1.60 | 0.408 | | |
| 4.00 p.m. | 13.77 | 3.766 | 3.66 | 1.69 | 0.461 | | |
| 12.00 " | 13.76 | 3.976 | 3.46 | 1.72 | 0.497 | | |
| June 2, 8.00 a.m. | 15.36 | 3.374 | 4.55 | 1.92 | 0.422 | | 20 gm. Merck's dextrose at 8.30 a.m. 15 tablets (No. 5) given in seven doses of 2 to 3 each, half hour intervals 8.30 to 11.30 a.m. |
| 4.00 p.m. | 13.28 | 1.694 | 8.43 | 1.66 | 0.212 | | |
| 12.00 " | 12.88 | 2.296 | 5.61 | 1.61 | 0.287 | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| June 3, 8.00 a.m. | 12.00 | 3.262 | 3.68 | 1.50 | 0.408 | | 20 gm. Merck's dextrose at 8.30 a.m. |
| 11.50 " | 8.68 | 1.162 | 7.46 | 2.26 | 0.303 | | |
| 2.00 p.m. | 0.74 | 0.056 | 13.21 | 0.34 | 0.026 | | At 2 p.m. very little urine in bladder, at 4 p.m. none. Complete suppression therefore began about noon. Dog in coma. Died during night. |

With this dog insulin was administered for the first time subcutaneously. There seems to be no doubt about the effect, although no control period with identical treatment except for insulin was obtained. This has been done in subsequent experiments. The insulin was given at 12.30 p.m. and because the dog went into convulsions soon after, 20 gm. Merck's dextrose were introduced into the stomach at 4.30 p.m. The dextrose upon analysis proved to be the pure monohydrate 90.8 per cent anhydrous glucose. Of the 18.16 gm. pure glucose administered only 11.6 gm. or roughly 65 per cent, were recovered. This result is reached by difference above the elimination for the two 8 hour periods following 11.50 p.m. The amount utilized may therefore be reckoned as (18.16 gm. − 11.86 gm. =) 6.30 gm. The dosage was 6 Rochester units or approximately 18 clinical units. The equivalent in glucose utilized per clinical unit is 0.35 gm. (see later computations). It should be noted that the blood sugar did not rise within 4 hours after giving the insulin.

After a day of rest the effect of oral administration was tried in direct comparison with the subcutaneous method. Tablet 5 was again used, seven doses of two or three each being given at intervals of half an hour from 8.30 to 11.30. The dog was in rather poor condition at 8.00 a.m. There was excessive salivation, the animal lying down and groaning continuously, not because of pain but apparently because of the disorganizing effect of phlorhizin on the nervous system. At noon, however, the dog was active and more rational. At 4.00 p.m. it was in still better condition but from 8 to 12 p.m. there was a recurrence of the same symptoms as seen in the morning, with the addition of tremors and running movements while lying down.

In this instance there was a tremendous effect on the excretion and presumably also on the utilization of sugar. For notwithstanding the administration of 18.16 gm. pure glucose at 8.30 a.m. there was a diminished rate of excretion per hour from 4 o'clock on to the next morning. Beyond this point we have no information for the dog was practically in a state of collapse and dextrose was again introduced into the stomach at 8.30 a.m. the next day in the hope of effecting restoration. This was largely regurgitated in a short while and the animal continued to grow worse, going into coma about 4.00 p.m. and dying during the night.

The animal's condition undoubtedly invalidates the exact figures attained at the height of the effect as the result of administering Tablet 5, and yet botb the nitrogen excretion and sugar excretion came back to the preinsulin level before the sugar was given on the 3rd. The entire reaction in contrast with the reaction to subcutaneous insulin is shown much more clearly on Chart 1. The difference is not typical as may be seen from later experiments.

In the chart it is seen that nitrogen and sugar fall in parallel fashion from the effect of subcutaneous administration. When glucose is given by stomach tube the rate of excretion naturally

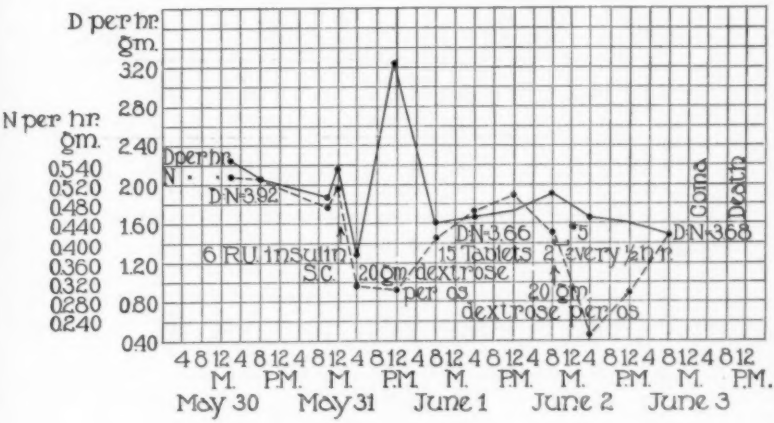


CHART 1. Dog 6. Phlorhizin.

rises enormously but the nitrogen is depressed still farther *as if* there were a real sparing effect. Next day (June 1) at 8.00 a.m. the two curves approach each other and at 4.00 p.m. the D:N ratio is restored to the complete diabetic level (curves come together). When the tablets were started and the dextrose given at 8.30 a.m. on the 2nd the two curves had separated somewhat (nitrogen low, D:N high), but from this point the descent of nitrogen was more rapid and reached a very low level, again indicating a sparing action. *Moreover the sugar excretion did not rise at all.*

This experiment seemed so encouraging that we proceeded at

TABLE VI.

*Dog 7.*

| Date. | Total D. | Total N. | D:N | D per hour. | N per hour. | R.Q. | Remarks. |
|---|---|---|---|---|---|---|---|
| *1924* | *gm.* | *gm.* | | *gm.* | *gm.* | | |
| June 9, 10.00 a.m. | 20.00 | 4.774 | 4.18 | 2.00 | 0.477 | | 25.24 gm. glucose at 10 a.m. |
| 11.43 a.m.–12.11 p.m. | | | | | | 0.671 | Dog quiet. |
| 12.11 p.m.–12.45 " | | | | | | 0.700 | " " |
| 12.45 " – 1.19 " | | | | | | 0.727 | " " |
| 6.00 " | 35.33 | 3.150 | 11.21 | 4.42 | 0.394 | | |
| 11.00 " | 9.52 | 1.988 | 4.78 | 1.90 | 0.398 | | |
| June 10, 8.00 a.m. | 21.98 | 3.262 | 6.73 | 2.44 | 0.362 | | |
| 10.00 a.m. | 4.16 | 0.798 | 5.21 | 2.08 | 0.399 | | 27.24 gm. glucose at 10 a.m. |
| Total 24 hrs. | 70.99 | | | | | | |
| 10.55–11.40 a.m. | | | | | | 0.661 | Dog quiet. |
| 11.40 a.m.–1.01 p.m. | | | | | | 0.755 | " restless and panting. |
| 1.01–2.00 p.m. | | | | | | 0.707 | " restless. |
| 2.00–3.03 " | | | | | | 0.721 | Few short spells of panting. |
| 4.00 p.m. | 32.05 | 2.233 | 14.35 | 4.52 | 0.372 | | Dog quiet. |
| 11.00 " | 10.52 | 1.736 | | | | | Loss of urine. |
| June 11, 8.00 a.m. | 14.79 | 2.898 | 5.10 | 1.64 | 0.322 | | 23 No. 5 tablets in seven doses at 8 to 10 a.m. |
| Total 24 hrs. | 61.52 | | | | | | |
| 10.00 a.m. | | | | | | | 27.24 gm. glucose. |
| 10.57–11.33 a.m. | | | | | | 0.735 | Dog quiet. |
| 11.33 a.m.–12.27 p.m. | | | | | | 0.677* | " " |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 11.33 a.m.–12.27 p.m. | | | | | | 0.677* | " " |
| June 11, 12.27–1.31 p.m. | | | | | | 0.769 | Dog quiet. |
| 1.31–2.28 " | | | | | | 0.680* | " " |
| 2.28–3.29 " | | | | | | 0.789 | " " |
| 4.20 p.m. | 36.62 | 3.010 | 12.16 | 4.39 | 0.361 | | |
| 11.00 " | 11.12 | 1.862 | 5.97 | 1.67 | 0.279 | | |
| June 12, 8.10 a.m. | 13.18 | 2.898 | 4.54 | 1.44 | 0.316 | | |
| Total 24 hrs., 10 min. | 60.92 | | | | | | |
| 8.10–10.05 a.m. | 3.87 | 0.819 | 4.72 | 2.02 | 0.429 | | 27.24 gm. glucose at 10.05 a.m. |
| 4.00 p.m. | | | | | | | Urine lost. |
| 4.00–11.00 p.m. | 11.83 | 2.184 | 5.41 | 1.69 | 0.312 | | |
| June 13, 10.00 a.m. | 22.40 | 3.622 | 6.18 | 2.03 | 0.329 | | 27.24 gm. glucose at 10.00 a.m. |
| 4.00 p.m. | 25.75 | 1.610 | 15.99 | 4.29 | 0.268 | | |
| 12.00 " | 12.70 | 2.016 | 6.30 | 1.59 | 0.252 | | |
| June 14, 8.00 a.m. | 10.41 | 2.282 | 4.55 | 1.30 | 0.285 | | |
| 8.45–9.22 a.m. | | | | | | 0.715 | |
| 9.22–9.47 " | | | | | | 0.723 | |
| 10.00 a.m. | 3.21 | 0.703 | 4.56 | 1.60 | 0.351 | | 27.24 gm. glucose at 10.20 a.m. |
| Total 24 hrs. | 52.07 | | | | | | 10 Rochester units E-29 subcutaneously at 10.30 a.m. |
| 11.31 a.m.–12.39 p.m. | | | | | | 0.738 | |
| 1.34–2.05 p.m. | | | | | | 0.704 | |
| 2.05–2.54 " | | | | | | 0.664 | |
| 4.00 p.m. | 15.80 | 1.555 | 10.16 | 2.63 | 0.259 | | Small loss of urine. |
| 12.00 " | 12.64 | 2.030 | 6.22 | 1.58 | 0.254 | | |

TABLE VI—*Concluded.*

| Date. | Total D. | Total N. | D:N | D per hour. | N per hour. | R.Q. | Remarks. |
|---|---|---|---|---|---|---|---|
| *1924* | *gm.* | *gm.* | | *gm.* | *gm.* | | |
| June 15, 10.00 a.m. | 15.04 | 3.066 | 4.90 | 1.50 | 0.306 | | No glucose or insulin. |
| Total 24 hrs. | 43.48 | | | | | | |
| June 16, 10.00 a.m. | 44.96 | 8.876 | 5.06 | 1.87 | 0.370 | | |
| 1.30 p.m. | | | | | | | 27.24 gm. glucose. 10 Rochester units E-29 subcutaneously. |
| 2.00 p.m. | 10.00 | 1.652 | 6.05 | 2.50 | 0.413 | | |
| 3.11–3.42 p.m. | | | | | | 0.656 | |
| 3.42–4.34 " | | | | | | 0.649 | |
| 4.34–5.30 " | | | | | | 0.721 | |
| 5.30–6.26 " | | | | | | 0.672 | |
| 10.00 p.m. | 21.60 | 2.261 | 9.55 | 2.70 | 0.283 | | |
| June 17, 10.00 a.m. | 22.82 | 3.334 | 6.84 | 1.90 | 0.278 | | |
| Total 24 hrs. | 54.42 | | | | | | |

* Leak in one train of absorbers.

once to repeat it with another dog employing the respiratory quotient as additional proof of utilization.

*Dog 7.*—A shaggy white wolfhound weighing 20 kilos started fasting on June 2. Phlorhizin given in two 1 gm. doses daily beginning June 4. Animal in excellent condition throughout the experiment. In spite of the heavy administration of phlorhizin the dog was not completely unable to burn sugar (as shown by the R.Q.) until June 9. On this day the D:N ratio at 10 a.m. was 4.18 but the R.Q. between 11.40 and 1.19 after the administration of 25.24 gm. sugar by stomach tube was in three successive periods, 0.67, 0.70, and 0.73. The R.Q. was determined with the aid of residual analyses in this and all subsequent experiments, *i.e.* after correction for changes in the composition of the chamber air. Even with this additional precaution an error occurs when the dog pants (see R.Q.'s for June 10).

Beginning on the 10th 30 gm. of Merck's dextrose, assaying 27.24 gm. glucose, were given each morning at 10.00 a.m. Insulin was administered in the form of the enteric coated Tablet 5 on the 11th and subcutaneously on the 14th and 16th. June 9, 10, 12, and 13 were taken as control days. The experiment is recorded graphically from the 10th to the 16th inclusive in Chart 2. Further details may be found in Table VI. Referring to the chart it may be seen that the sugar excretion reached a slightly higher point on the 10th than when insulin was given on the 11th; also that the R.Q. rose to levels which indicate clearly the combustion of sugar in small quantity. (A line drawn from the peak of the sugar curve on June 10 to the peak on the 13th shows the rate of decline in basal metabolism due to fasting.) The second control day failed of its complete purpose because of the loss of urine at 4.00 p.m., hence was repeated with determination of the R.Q.'s on the 13th. When insulin was given subcutaneously on the 14th the sugar excretion at 4.00 p.m. was very much lower. There was a small loss of urine at this time but not enough to affect the result materially. The R.Q.'s on this day did not reach so high a level as after the oral administration on the 11th, and the effect of the insulin was soon spent, for the R.Q. came back in the second and third periods to diabetic level. This rapid return to diabetic level has frequently been seen also with depancreatized dogs treated with insulin (2). On the 15th no sugar or insulin was given

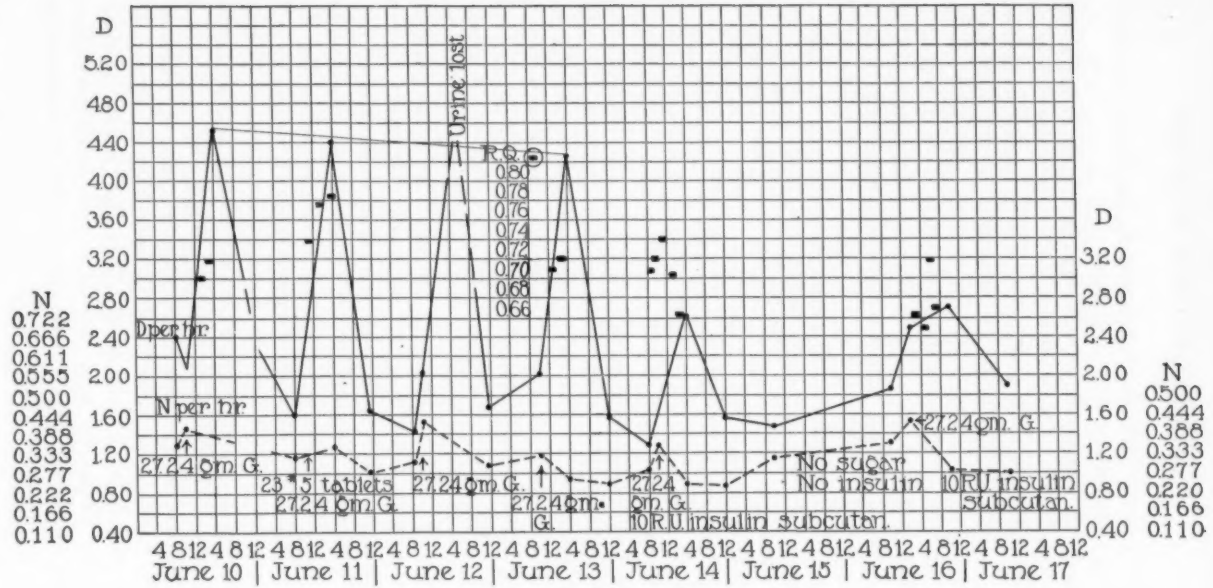


**Chart 2. Dog 7. No food.**

but on the 16th the same dosage of both was repeated as on the 14th, with very much the same result as regards excretion but with little, if any, effect on the R.Q. This is because the respiration experiment was not begun until nearly 5 hours after insulin and glucose were administered. Hawley and Murlin (11) have shown that the typical effect of insulin on the R.Q. of normal rabbits terminates within 3 hours.

Our interpretation of the experiment is that insulin given subcutaneously to the phlorhizinized dog may cause a rapid increase in the combustion of sugar for a short time only. The effect on excretion is much more prolonged (see *e.g.*, June 14). Given in suitable form in an enteric coated tablet the rise of quotient may be greater but the effect on excretion is not so prolonged. This particular tablet contained malic acid and in the twenty-three tablets there would be approximately 5 gm. A part of this acid at least, as we have found quite recently, is converted to sugar; hence the small effect on excretion. There was no sign of a nephrotoxic effect such as is described by Rose (12) in the rabbit after much larger doses.

On account of the loss of urine by accident on several occasions, the daily total excretions of sugar are not particularly convincing. Collection was complete on the 9th, 11th, 13th, and 15th. The total excretion on these 4 days was 70.99, 60.92, 52.07, and 44.96 gm., respectively. The decline is nearly uniform. However, the amount of glucose ingested was 25.24 gm. on the 9th and 27.24 gm. on each of the other days. Insulin was given in tablet form on the 11th. The excretion on this day is about 1 gm. less than the mean between the 9th and 13th, notwithstanding the smaller feeding on the 9th, and notwithstanding the conversion of malic acid to sugar. The showing in terms of glucose utilization is not great, but taken in conjunction with the higher R.Q. can only be explained as a genuine effect of insulin absorbed from the alimentary tract. The subcutaneous effect cannot be calculated.

CHART 2. Dog 7. No food.

*Dog 8.*—Weight 10 kilos. Received 1 gm. phlorhizin in olive oil daily. No food. Remained in good condition throughout. Urine collection started on the 4th day of fasting and the 2nd of phlorhizin with the D:N ratio at 3.48. Next day, the 3rd of phlorhizin, the R.Q. was determined, starting 1 hour and 20 minutes after the introduction of 20 gm. of glucose into the stom-

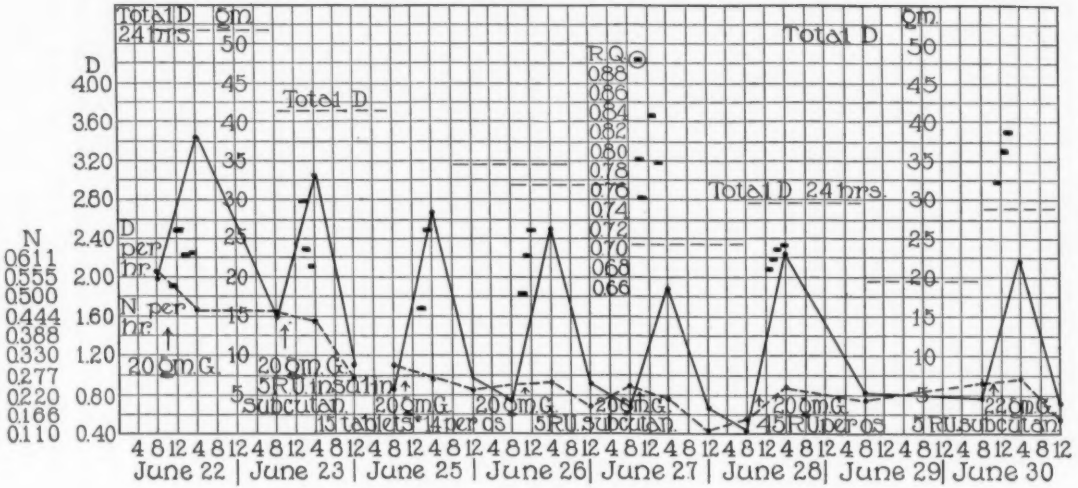


CHART 3. Dog 8. No food.

ach. The result with the possible exception of one period, proved the dog to be quite unable to oxidize glucose. When insulin was given subcutaneously next day there was one high R.Q. (0.89) at a time corresponding to the highest of the control day but because of the very low R.Q. which followed this (0.64) the mean of the two (0.76) is shown graphically as the maximum effect. No leak was found in the apparatus. Excretion of sugar was 11 gm. less than on the previous control day (see Summary, Table VII), and the hourly excretion of both dextrose and nitrogen fell to a much lower level at the end of the day (see Chart 3, 12 p.m.).

A new tablet, No. 14, containing approximately twelve parts of sodium oleate to one part of a fairly pure insulin powder (amyl alcohol precipitate) was given on the 24th but the R.Q. was omitted in order to follow the curve of blood sugar. The tablet apparently had some effect on the total sugar excretion though the amount is uncertain, for no control day was introduced until the 26th when the total was 33.04 gm. Taking the drop in 4 days (23rd to 27th) as 20 gm., the excretion on the 24th should have been about 43 gm. Actually it was just under 40. The blood sugar curve reaches its maximum at 12.00 noon, just 2 hours after administration.

The tablet given again on the 25th shows a slight effect on the hourly excretion of sugar (Chart 3) and an indeterminate effect on total excretion (35.5 gm. instead of a predictable amount of probably 38 gm.), but only a slight effect on the R.Q. The tablets were given from 8 to 10 a.m. while the R.Q. was not started until 1.15. In the first period a leak was found in the apparatus, but all conditions were good in the second and a quotient at least four points above the average diabetic level was obtained. This tablet, however, did not produce much effect in other experiments (not here recorded) and it is practically certain that the insulin was largely inactivated by the large amount of alkali set free by the high proportion of soap. Test-tube experiments have shown that five parts of the same soap will inactivate insulin within a few hours when dissolved in water. The fact remains however that the effect of 5 Rochester units of a thoroughly tested insulin given subcutaneously on the 23rd did not produce any greater effect on excretion up to 4 p.m. than did the fifteen tablets given orally on the 25th.

TABLE VII.

*Dog 8.*

| Date. | Total D. | Total N. | D:N | D per hr. | N per hr. | Blood sugar. | R.Q. | Remarks. |
|---|---|---|---|---|---|---|---|---|
| *1924* | *gm.* | *gm.* | | *gm.* | *gm.* | *mg.* | | |
| June 21, 4.00 p.m.–12.00 p. m. | 14.13 | 4.053 | 3.48 | 1.77 | 0.507 | | | 4th day of fasting. 2nd day of phlorhizin. |
| June 22, 8.00 a.m. | 15.87 | 4.557 | 3.48 | 1.98 | 0.569 | | | 20 gm. glucose at 10 a.m. |
| 11.20 a.m.–12.33 p.m. | | | | | | | 0.672 | |
| 12.33–1.16 p.m. | | | | | | | 0.727 | |
| 1.16–2.02 " | | | | | | | 0.699 | |
| 2.02–2.38 " | | | | | | | 0.707 | |
| 4.00 p.m. | 27.78 | 3.682 | 7.54 | 3.47 | 0.460 | | | |
| June 23, 8.00 a.m. | 25.64 | 7.224 | 3.54 | 1.60 | 0.451 | | | 20 gm. glucose 10 a.m. 5 Rochester units No. 16, subcutaneously, 10 a.m. |
| Total 24 hrs. | 53.42 | | | | | | | |
| 11.30 a.m.–12.12 p.m. | | | | | | | 0.704 | |
| 12.12–1.12 p.m. | | | | | | | 0.887 | Probably correct values. |
| 1.12–2.12 " | | | | | | | 0.639 | Mean 0.76. |
| 2.12–2.56 " | | | | | | | 0.716 | |
| 2.56–3.34 " | | | | | | | 0.690 | |
| 4.00 p.m. | 24.69 | 3.472 | 7.07 | 3.08 | 0.434 | | | |
| 12.00 " | 8.90 | 2.142 | 4.15 | 1.11 | 0.268 | | | |
| June 24, 8.00 a.m. | 8.90 | 2.604 | 3.41 | 1.11 | 0.325 | | | 8–10 a.m. five doses of 3 tablets each, No. 14. |
| Total 24 hrs. | 42.49 | | | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Total 24 hrs. | 42.49 | | | | | | | tablets each, No. 14. |
| 9.00 a.m. | | | | | | 42 | | |
| 10.00 " | | | | | | 42 | | 20 gm. glucose at 10 a.m. |
| 11.00 " | | | | | | 118 | | |
| 12.00 m. | | | | | | 166 | | |
| 1.00 p.m. | | | | | | 120 | | |
| 2.00 " | | | | | | 90 | | |
| 3.00 " | | | | | | 67 | | |
| 4.00 " | 25.35 | 2.562 | 9.89 | 3.17 | 0.320 | 59 | | |
| 5.00 " | | | | | | 54 | | |
| 6.00 " | | | | | | 50 | | |
| 12.00 " | 7.85 | 2.226 | 3.52 | 0.98 | 0.278 | | | |
| June 25, 8.00 a.m. | 6.78 | 2.408 | 2.81 | 0.85 | 0.301 | | | 8–10 a.m. 5 doses of 3 tablets each, No. 14, 20 gm. glucose at 10 a.m. |
| Total 24 hrs. | 39.98 | | | | | | | |
| 1.15–1.56 p.m. | | | | | | | 0.639 | Valve leak. |
| 1.56–3.10 " | | | | | | | 0.734 | |
| 4.00 p.m. | 21.50 | 2.142 | 10.03 | 2.69 | 0.268 | | | |
| 12.00 " | 7.84 | 1.876 | 4.18 | 0.98 | 0.234 | | | |
| June 26, 8.00 a.m. | 6.15 | 2.072 | 2.96 | 0.77 | 0.259 | | | 20 gm. glucose at 10 a.m. |
| Total 24 hrs. | 35.49 | | | | | | | |
| 9.53–10.22 a.m. | | | | | | | 0.661 | |
| 10.22–10.57 " | | | | | | | 0.701 | |
| 10.57–11.41 " | | | | | | | 0.729 | |
| 4.00 p.m. | 20.25 | 2.170 | 9.33 | 2.53 | 0.271 | | | |
| 12.00 " | 7.54 | 1.498 | 5.03 | 0.94 | 0.187 | | | |

TABLE VII—*Concluded.*

| Date. | Total D. | Total N. | D:N | D per hr. | N per hr. | Blood sugar. | R.Q. | Remarks. |
|---|---|---|---|---|---|---|---|---|
| *1924* | *gm.* | *gm.* | | *gm.* | *gm.* | *mg.* | | |
| June 27, 8.00 a.m. | 5.25 | 2.016 | 2.60 | 0.66 | 0.252 | | | 10 a.m. 20 gm. glucose and 5 Rochester units No. 16 subcutaneously. |
| Total 24 hrs. | 33.04 | | | | | | | |
| 11.02–11.41 a.m. | | | | | | | 0.804 | |
| 11.41 a.m.–12.22 p.m. | | | | | | | 0.760 | |
| 12.22–1.01 p.m. | | | | | | | 0.853 | |
| 1.01–1.57 " | | | | | | | 0.787 | |
| 4.00 p.m. | 15.40 | 1.736 | 8.87 | 1.92 | 0.217 | | | |
| 12.00 " | 5.40 | 0.931 | 5.80 | 0.67 | 0.116 | | | |
| June 28, 8.00 a.m. | 3.71 | 1.232 | 3.01 | 0.46 | 0.154 | | | 45 Rochester units No. 16 in 6 salol-coated tablets. 2 tablets each at 9, 9.30, 10 a.m. |
| Total 24 hrs. | 24.51 | | | | | | | |
| 12.02–12.44 p.m. | | | | | | | 0.689 | 20 gm. glucose at 10 a.m. |
| 12.44– 1.40 " | | | | | | | 0.706 | |
| 1.40– 2.47 " | | | | | | | 0.719 | |
| 2.47– 3.32 " | | | | | | | 0.714 | |
| 4.00 p.m. | 17.70 | 1.834 | 9.65 | 2.21 | 0.229 | | | |
| June 29, 8.00 a.m. | 12.89 | 3.360 | 3.83 | 0.81 | 0.210 | | | No glucose or insulin. |
| Total 24 hrs. | 30.59 | | | | | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Total 24 hrs. | | 30.59 | | | | | | | |
| June 30, | 8.00 a.m. | 19.05 | 6.160 | 3.09 | 0.79 | 0.256 | | | 22 gm. glucose at 10.00 a.m. 5 Rochester units No. 16 subcutaneously at 10 a.m. |
| | 11.33 a.m.–12.10 p.m. | | | | | | | 0.784 | |
| | 12.10–12.45 p.m. | | | | | | | 0.817 | |
| | 12.45– 1.20 " | | | | | | | 0.834 | |
| | 1.20– 2.00 " | | | | | | | 0.702 | |
| | 4.00 p.m. | 17.40 | 2.170 | 8.02 | 2.17 | 0.271 | | | |
| | 12.00 " | 5.89 | 1.204 | 4.89 | 0.74 | 0.150 | | | |
| July 1, | 8.00 a.m. | 4.80 | 1.848 | 2.60 | 0.60 | 0.231 | | | 22 gm. glucose at 10.00 a.m. |
| | 4.00 p.m. | 16.01 | 1.722 | 9.30 | 2.00 | 0.215 | | | |
| July 2, | 8.00 a.m. | 9.54 | 2.870 | 3.32 | 0.60 | 0.179 | | | |

Summary.

| Date. | Total D. | Remarks. |
|---|---|---|
| *1924* | *gm.* | |
| June 22 | 53.42 | 20 gm. glucose. |
| " 23 | 42.49 | 20 " " 5 Rochester units No. 16 subcutaneously. |
| " 24 | 39.98 | 20 " " 15 tablets No. 14 orally. |
| " 25 | 35.49 | 20 " " 15 " " 14 " |
| " 26 | 33.04 | 20 " " |
| " 27 | 24.51 | 20 " " 5 Rochester units No. 16 subcutaneously. |
| " 28 | 30.59 | 20 " " 45 " " " 16 in salol tablets. |
| " 29 | 19.05 | No glucose or insulin. |
| " 30 | 28.09 | 22 gm. glucose. 5 Rochester units No. 16 subcutaneously. |
| July 1 | 25.55 | 22 " " |

TABLE VIII.

*Dog 9.*

| Date. | Total D. | Total N. | D:N | D per hr. | N per hr. | R.Q. | Remarks. |
|---|---|---|---|---|---|---|---|
| *1924* | *gm.* | *gm.* | | *gm.* | *gm.* | | |
| July 13, 10.00 a.m. | | | | | | | 8th day fasting. 5th day phlorhizin. 30 gm. glucose at 10.00 a.m. |
| 4.00 p.m. | | | | | | | Urine lost. |
| 10.00 " | 14.95 | 2.674 | 5.55 | 2.49 | 0.446 | | |
| July 14, 10.00 a.m. | 27.05 | 6.958 | 3.88 | 2.25 | 0.579 | | 12 Rochester units E-29-30-S subcutaneously at 10.15 a.m. 30 gm. glucose at 10.00 a.m. |
| 11.18 a.m.–12.00 m. | | | | | | 0.773 | |
| 12.00–1.03 p.m. | | | | | | 0.705 | |
| 1.03–2.00 " | | | | | | 0.718 | |
| 4.00 p.m. | 36.40 | 2.996 | 12.15 | 6.06 | 0.499 | | |
| 10.00 " | 12.59 | 2.205 | 5.71 | 2.10 | 0.367 | | |
| July 15, 10.00 a.m. | 31.75 | 5.936 | 5.34 | 2.65 | 0.494 | | 30 gm. glucose at 10.00 a.m. |
| 11.22 " –12.08 p.m. | | | | | | 0.690 | |
| 12.08–1.02 p.m. | | | | | | 0.697 | |
| 1.02–2.12 " | | | | | | 0.676 | |
| 4.00 p.m. | 39.20 | 2.674 | 14.65 | 6.53 | 0.446 | | |
| 10.00 " | 16.10 | 2.688 | 5.98 | 2.68 | 0.448 | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 10.00 " | 16.10 | 2.688 | 5.98 | 2.68 | 0.448 | | |
| July 16, 10.00 a.m. | 32.00 | 5.712 | 5.60 | 2.66 | 0.476 | | 12 Rochester units No. 16 subcutaneously at 10.00 a.m. 30 gm. glucose at 10.00 a.m. |
| 11.24 a.m.–12.10 p.m. | | | | | | 0.770 | |
| 12.10–1.13 p.m. | | | | | | 0.688 | |
| 1.13–1.55 " | | | | | | 0.702 | |
| 4.00 p.m. | 25.80 | 2.128 | 12.12 | 4.30 | 0.355 | | |
| 10.00 " | 15.50 | 1.736 | 8.93 | 2.58 | 0.289 | | |
| July 17, 10.00 a.m. | 26.70 | 4.494 | 5.94 | 2.22 | 0.374 | | 30 gm. glucose at 10.00 a.m. |
| 11.21 " –12.23 p.m. | | | | | | | |
| 4.00 p.m. | 32.70 | 2.240 | 14.59 | 5.45 | 0.373 | | |
| 10.00 " | | | | | | | Violent convulsions. Animal killed. |

The control day, June 26, and the insulin day, June 27, are perfectly clear-cut. In this case the effect of insulin on the R.Q. continued for at least 4 hours. The effect on excretion of sugar is indicated by the lower peak in the graph for dextrose per hour. Since the animal weighed 10 kilos at the beginning of the experiment the dosage is equivalent to 1 rabbit unit for each 2 kilos of weight. It is evident that this dosage is quite sufficient to affect both the excretion and the combustion of sugar very materially in the phlorhizinized animal. The D:N ratios however were low.

On the 28th insulin was again given *per os*. There was no effect on the respiratory quotient up to 4 hours after administration. The effect on excretion seems to have been to increase the output of sugar slightly at the end of 6 hours and to increase the output of nitrogen also to a slight extent. Assuming that there was no effect on this day, that it may be regarded as a control day, the decline in 24 hour elimination on the 27th becomes 7.30 gm., equal to 0.48 gm. per clinical unit of insulin. Calculation of the combustion of sugar from the nitrogen excretion and the respiratory exchange shows that 3.72 gm. were oxidized in 3 hours (from 11.02 to 1.57, see Table VII). While the R.Q. had not returned to the diabetic level at the end of this time it was well on the decline and it is practically certain that some of the sugar retained would remain unaccounted for by combustion.

On the 29th no insulin or sugar was given. On the 30th 22 gm., instead of 20 gm., were administered with insulin at the usual time, 10.00 a.m., and the R.Q. was studied from 11.30 until 2.00 o'clock. The quotient was distinctly elevated for three periods and then returned to the diabetic level quite suddenly in the last period. Curiously enough it is difficult to make out any effect on excretion in the first 6 hours. This is partly, if not wholly, due to the fact that by mistake 2 gm. more of glucose were given at the same time as the insulin. The whole experiment indicates clearly that insulin given subcutaneously to a phlorhizined animal which has been wholly unable to oxidize sugar may affect the combustion of sugar for a period of 4 to 6 hours. In the previous experiment the effect seemed to wear off much earlier than this when the insulin was given subcutaneously in about the same dose.

*Dog 9.*—This was a large, female hound weighing 20 kilos. The

dog had been used during the month of June for experiments with adrenalin, having received 21 mg. of adrenalin daily on June 4, 10, 16, and 23, glucose being administered *per os* in the amount of 36.3 gm. each day. The animal also had received several doses of Tablet 5 on June 4 and Tablet 9, containing hydrochloric acid, on the 16th. Fasting was started on July 6, phlorhizin on July

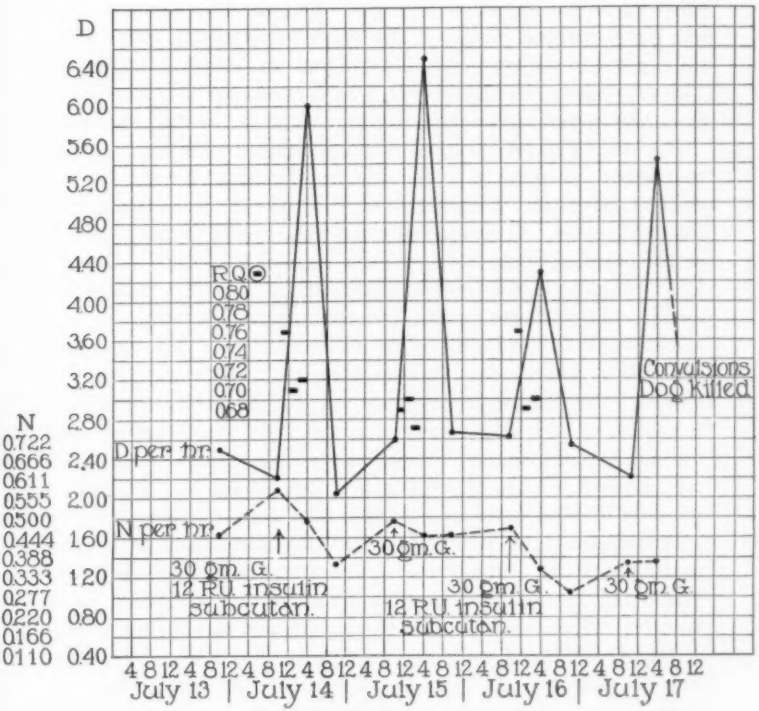


CHART 4. Dog 9. No food.

9, 2 gm. each day. All aseptic precautions were observed. Collection of urine began on July 11 but inaccuracy of collection was discovered and corrected at 4.00 p.m. on July 13, from which time Table VIII begins. On July 14, the D:N ratio being at a satisfactory level, insulin was administered subcutaneously at

10.15 a.m., 30 gm. C.P. glucose being administered by stomach tube at the same time.

Chart 4 shows graphically the effect upon the R.Q. and the effect upon the excretion of sugar in contrast with July 15 when the same amount of glucose but no insulin was given. Again on the 16th the same dosage of insulin administered subcutaneously showed an abrupt but moderate rise in R.Q. and also a profound effect upon the excretion of sugar. On the 17th again glucose was given without insulin. The animal was seized with violent convulsions in the evening of July 17 and was killed at 10.00 p.m. This experiment, like that on Dog 7, showed only a brief effect of the administered insulin upon the R.Q. The D:N ratios were high.

*Dog 10.*—Female mongrel collie weighing 10 kilos. Fasting was begun on July 16, phlorhizin on the 18th, dosage 1.2 gm. daily in olive oil. Dog remained in good condition throughout the experiment. Collection of urine was started on the 19th and on the morning of the 20th the D:N ratio of 3.4 was found (Table IX). 20 gm. glucose were administered at 10.00 a.m. and of this amount only 13.2 gm. were recovered, assuming the same rate of excretion next morning as before administration. No respiratory experiment was made. On the 21st, after the same dosage of glucose, a respiratory experiment proved the animal practically incapable of oxidizing sugar. On the 22nd collection of urine during the night was incomplete but the catheter specimen at 10.00 a.m. showed a D:N ratio of 3.45. Ten tablets No. 16 had been given in divided doses from 7.00 to 9.30 a.m. Glucose was then given as usual at 10.00 a.m. and the respiration experiment started at 10.50. There was no effect on the R.Q. until the last hour from 1.00 to 2.00 when the quotient rose suddenly, nearly to 0.77. With this exception the experiment resulted negatively.

*Dog 11.*—Animal weighing 12.7 kilos. Fasting was begun on July 23, phlorhizin, 1.5 gm. in a single dose per day, on the 25th. Animal in excellent condition throughout the observation. Collection of urine was started on the 27th at 10.00 p.m. and on the morning of the 28th the D:N was 3.8. The respiration experiment on this day proved the animal incapable of oxidizing glucose. On the 29th, with the D:N ratio at 3.51, insulin was administered in the form of tablets coated with stearic acid. The insulin preparation contained 1 per cent HCl in addition to the insulin powder. The respiration experiment, begun at 10.50 and ending at 3.00

TABLE IX.
*Dog 10.*

| Date. | Total D. | Total N. | D:N | D per hr. | N per hr. | R.Q. | Remarks. |
|---|---|---|---|---|---|---|---|
| *1924* | *gm.* | *gm.* | | *gm.* | *gm.* | | |
| July 19, 10.00 p.m. | | | | | | | Catheterized, urine discarded. |
| July 20, 10.00 a.m. | 13.18 | 3.878 | 3.39 | 1.10 | 0.323 | | 20 gm. glucose at 10.00 a.m. |
| 4.00 p.m. | 18.90 | 1.792 | 10.54 | 3.15 | 0.299 | | |
| 10.00 " | 8.00 | 1.344 | 5.95 | 1.33 | 0.224 | | |
| July 21, 10.00 a.m. | 12.90 | 3.129 | 4.12 | 1.08 | 0.261 | | 20 gm. glucose at 10.00 a.m. |
| 11.09 " –12.10 p.m. | | | | | | 0.677 | |
| 12.10–1.05 p.m. | | | | | | 0.738 | |
| 1.05–2.00 " | | | | | | 0.675 | |
| 4.00 p.m. | 17.40 | 1.442 | 12.06 | 2.90 | 0.240 | | |
| 10.00 " | 7.28 | 1.120 | 6.50 | 1.21 | 0.186 | | |
| July 22, 10.00 a.m. | 4.76 | 1.379 | 3.45 | | | | Catheter urine only. 7.00–9.30 a.m., 10 tablets No. 16. 20 gm. glucose at 10.00 a.m. |
| 10.51–11.55 a.m. | | | | | | 0.675 | |
| 11.55 a.m.–1.05 p.m. | | | | | | 0.703 | |
| 1.05–2.02 p.m. | | | | | | 0.765 | |
| 4.00 p.m. | 16.00 | 1.340 | 11.94 | 2.66 | 0.223 | | |
| 10.00 " | 7.18 | 1.130 | 6.36 | 1.20 | 0.188 | | |
| July 23, 10.00 a.m. | 12.50 | 2.723 | 4.59 | 1.04 | 0.227 | | Dog accidentally killed. |
| 10.15 " | | | | | | | |

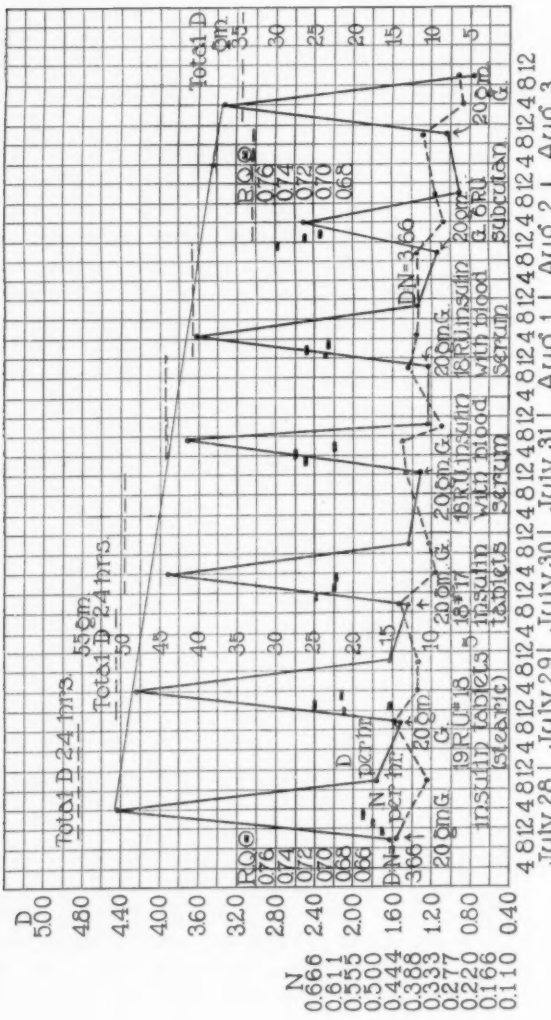
Total D 24 hrs. 55 Gm.
Total D 24 hrs.
RQ
DN-3.66
20 mG. G.
18 RU insulin with blood serum
20 Gm. G. subcutan.
July 28 | July 29 | July 30 | July 31 | Aug. 1 | Aug. 2 | Aug. 3

CHART 5. Dog 11. No food.

o'clock, exhibits no change in the average R.Q. The curve of excretion also indicates no change. On the 30th insulin was given in the form of another tablet containing an amino acid in addition to the insulin powder and in equal amount. This amino acid was obtained from Professor Lafayette B. Mendel, who states that on solution in water it releases 20 per cent of its weight in the form of hydrochloric acid. It was hoped that this amount of acid would suffice to inactivate trypsin. The curve of excretion (Chart 5) shows that there was a slight effect of the insulin but the R.Q. exhibits no material improvement in the capacity to oxidize sugar.

A different mode of inactivation was tried on the next 2 experimental days. Insulin in the form of salt precipitate was mixed with 20 gm. glucose and with 70 cc. centrifuged blood serum, the entire mixture being given by stomach tube at 12.20. The respiration experiment was begun at 1.15 and continued until 4.00 o'clock. There seems to be a slight effect of this mode of administration upon the absorption of insulin for the R.Q. was distinctly elevated in the first two periods, only to return to its diabetic level in the 3rd. The curve of excretion indicates also slightly less rapid elimination of glucose than on the average for the control days (shown by sloping line at apices of curve). Because of the protein contained in the blood serum (N equals 0.809 gm.) and perhaps also the small amount of protein in the relatively crude insulin employed, there would be 2 or 3 gm. more sugar to be excreted. The same experiment was tried again on August 1, 200 cc. of blood serum being used with the same amount of glucose and of insulin powder as before. There was not quite so great an effect on the R.Q. but the curve of excretion, making allowance for 8 gm. additional sugar to be excreted from the blood proteins, indicates a less rapid elimination of glucose at the 6 hour period. None of the three methods used for alimentary administration of insulin was strikingly successful with this animal. The first depended upon stearic acid as a coating which should resist the stomach digestion and preserve the contained insulin until it should be set free in the intestine. Experiments on other animals with the stearic acid coating had demonstrated that the coating is readily digested under the influence of bile and trypsin. However, those experiments also demonstrated that when the first tablet thus coated, and containing a considerable

TABLE X.
*Dog 11.*

| Date. | Total D. | Total N. | D:N | D per hr. | N per hr. | R.Q. | Remarks. |
|---|---|---|---|---|---|---|---|
| *1924* | *gm.* | *gm.* | | *gm.* | *gm.* | | |
| July 27, 10.00 p.m. | | | | | | | Catheterized. |
| July 28, 10.00 a.m. | 20.00 | 5.264 | 3.80 | 1.66 | 0.439 | | 5th day fasting. 3rd day phlorhizin. 20 gm. glucose at 10.00 a.m. |
| 11.06–11.47 a.m. | | | | | | 0.646 | |
| 11.47 a.m.–1.00 p.m. | | | | | | 0.663 | |
| 1.00–2.00 p.m. | | | | | | 0.675 | |
| 4.00 p.m. | 26.70 | 2.352 | 11.35 | 4.45 | 0.392 | | |
| 10.00 " | 10.75 | 2.030 | 5.29 | 1.79 | 0.338 | | |
| July 29, 10.00 a.m. | 18.68 | 5.320 | 3.51 | 1.56 | 0.443 | | 7.00–9.30 a.m. 19 Rochester units insulin preparation No. 18 stearic acid-coated. 20 gm. glucose at 10.00 a.m. |
| Total for 24 hrs. | 56.13 | | | | | | |
| 10.52–11.50 a.m. | | | | | | 0.696 | |
| 11.50 a.m.–12.53 p.m. | | | | | | 0.637 | |
| 12.53–2.00 p.m. | | | | | | 0.723 | |
| 2.00–3.03 " | | | | | | 0.691 | |
| 4.00 p.m. | 25.50 | 2.212 | 11.53 | 4.25 | 0.369 | | |
| 10.00 " | 10.00 | 2.170 | 4.61 | 1.66 | 0.362 | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| July 30, 10.00 a.m. | 17.38 | 5.264 | 3.30 | 1.45 | 0.439 | | 8.00–9.30 a.m. 18 tablets No. 17. 20 gm. glucose at 10.00 a.m. |
| Total for 24 hrs. | 52.88 | | | | | | |
| 11.27 a.m.–12.31 p.m. | | | | | | 0.716 | |
| 12.31–1.33 p.m. | | | | | | 0.700 | |
| 1.33–2.24 " | | | | | | 0.698 | |
| 4.00 p.m. | 23.40 | 1.960 | 11.93 | 3.90 | 0.327 | | |
| 10.00 " | 8.79 | 2.030 | 4.33 | 1.46 | 0.338 | | |
| July 31, 12.20 p.m. | 19.45 | 5.768 | 3.37 | 1.36 | 0.402 | | 18 Rochester units salt ppt. 20 gm. glucose in 70 cc. blood serum at 12.20 p.m. (0.809 gm. N).* |
| Total for 26 hrs., 20 min. | 51.64 | | | | | | |
| Estimated for 24 hrs. | 49.6 | | | | | | |
| 1.15–2.47 p.m. | | | | | | 0.725 | |
| 2.47–3.25 " | | | | | | 0.737 | |
| 3.25–4.03 " | | | | | | 0.701 | |
| 6.20 p.m. | 22.20 | 2.114 | 10.50 | 3.70 | 0.352 | | |
| 10.00 " | 4.44 | 1.092 | 4.06 | 1.21 | 0.298 | | |
| Aug. 1, 10.30 a.m. | 15.38 | 4.942 | 3.11 | 1.23 | 0.395 | | 18 Rochester units salt ppt. 20 gm. glucose in 200 cc. blood serum at 10.30 a.m. (2.312 gm. N).* |
| Total for 22 hrs., 10 min. | 42.02 | | | | | | |
| Estimated for 24 hrs. | 43.8 | | | | | | |
| 11.22 a.m.–12.40 p.m. | | | | | | 0.713 | |
| 12.40–1.25 p.m. | | | | | | 0.725 | |
| 1.25–2.34 " | | | | | | 0.706 | |
| 4.30 p.m. | 21.74 | 2.268 | 9.58 | 3.62 | 0.378 | | |
| 10.30 " | 8.18 | 2.226 | 3.67 | 1.36 | 0.371 | | |

TABLE X—*Concluded.*

| Date. | Total D. | Total N. | D:N | D per hr. | N per hr. | R.Q. | Remarks. |
|---|---|---|---|---|---|---|---|
| *1924* | *gm.* | *gm.* | | *gm.* | *gm.* | | |
| Aug. 2, 10.00 a.m. | 13.35 | 4.382 | 3.04 | 1.16 | 0.381 | | 20 gm. glucose at 10.00 a.m. 6 Rochester units E-29-30-S subcutaneously at 10.00 a.m. |
| Total for 23 hrs., 30 min. | 43.27 | | | | | | |
| 10.40–11.23 a.m. | | | | | | 0.756 | |
| 11.23–11.51 " | | | | | | 0.727 | |
| 11.51 a.m.–12.53 p.m. | | | | | | 0.715 | |
| 4.00 p.m. | 15.40 | 1.715 | 8.98 | 2.56 | 0.286 | | |
| 10.00 " | 5.56 | 1.834 | 3.03 | 0.93 | 0.306 | | |
| Aug. 3, 10.00 a.m. | 12.81 | 4.298 | 2.98 | 1.07 | 0.358 | | 20 gm. glucose at 10.00 a.m. |
| Total for 24 hrs. | 33.77 | | | | | | |
| 4.00 p.m. | 20.00 | 1.407 | 14.21 | 3.33 | 0.234 | | |
| Aug. 4, 10.00 a.m. | 14.00 | 4.396 | 3.18 | 0.77 | 0.244 | | Dog approaching convulsions. Killed at 10.15 a.m. |
| Total for 24 hrs. | 34.00 | | | | | | |

*Blood serum used: 1.156 gm. nitrogen per 100 cc.

amount of organic acid, is disintegrated the resulting acidity of the intestinal contents restricts further digestion or solution of the coating. This particular tablet contained inorganic acid (see above). Tablet 17 used on the 30th was coated with a salol preparation and with gelatin on the outside. No control tests were made in the intestines of other animals but there is reason to believe from results with other tablets, that this one was disintegrated. The blood serum method was undertaken in the hope that the antitryptic effect of blood serum might be sufficient to protect the insulin from its destructive action. Epstein (13) has recently brought forward facts which indicate that the destructive action of pancreatic juice is not due, primarily, to the proteolytic action of trypsin, but rather to its rennin-like action. The results with Dog 11 seem to offer some hope of successful protection of insulin against the destructive effects of the intestinal contents whatever they may be.

In contrast with these results so far as the effect upon excretion is concerned stands the experiment of August 2. 6 rabbit units of a purified insulin given subcutaneously at 10.00 a.m. produced a very marked effect on the excretion of sugar but produced only a little larger rise in the R.Q., namely to 0.76. The quotient returned gradually in the next two periods almost to the diabetic level. A control period on the 3rd terminated the experiment. A line connecting the peak of the curve of excretion on this control day with the peak of the curve on July 28, indicates the decline from day to day due to fasting. Taking this as a criterion of excretion and making allowance for the sugar which must have come from the protein contained in the blood serum, there is unmistakably a positive effect of the insulin on July 31 and August 1, which would substantiate the slight increase in the R.Q. observed on those days.

The apparently much greater effect of insulin on the excretion of sugar produced by subcutaneous than by oral administration, while the effect on the R.Q. is but little better, is a little misleading; for when the total excretion for 24 hours[1] (see Chart 5

[1] Unfortunately the dog was not catheterized at 10.00 a.m., the usual time, on July 31, but at 12.20 p.m. However, it is possible to estimate pretty accurately the total not only for this day but for the 24 hours ending at 10.30 a.m. next day. These estimated totals are shown in Chart 5, the actual totals in Table X.

TABLE XI.

*Dog 12.*

| Date. | Total D. | Total N. | D:N | D per hr. | N per hr. | R.Q. | Remarks. |
|---|---|---|---|---|---|---|---|
| *1924* | *gm.* | *gm.* | | *gm.* | *gm.* | | |
| Aug. 12, 12.00 m. | | | | | | | Catheterized, urine discarded. 3rd phlorhizin day. 500 gm. beef heart at 12.00 noon. |
| 2.00–3.00 p.m. | | | | | | 0.670 | |
| 3.00–4.03 " | | | | | | 0.719 | |
| 4.03–4.48 " | | | | | | 0.661 | |
| 8.00 p.m. | 15.38 | 4.200 | 3.66 | 1.92 | 0.525 | | |
| Aug. 13, 10.00 a.m. | 26.00 | 8.330 | 3.12 | 1.62 | 0.520 | | 500 gm. beef heart at 10.00 a.m. |
| 12.13–1.04 p.m. | | | | | | 0.673 | |
| 1.04–2.00 " | | | | | | 0.706 | |
| 2.00–2.59 " | | | | | | 0.726 | (Activity.) |
| 6.00 p.m. | 17.70 | 5.810 | 3.04 | 2.21 | 0.726 | | |
| Aug. 14, 10.00 a.m. | 25.35 | 8.820 | 2.87 | 1.58 | 0.551 | | 500 gm. beef heart at 10.00 a.m. 5 Rochester units E-29-30-S subcutaneously at 12.00 noon. |
| Total for 24 hrs. | 43.05 | | | | | | |
| 12.27–1.30 p.m. | | | | | | 0.751 | |
| 1.30–2.33 " | | | | | | 0.698 | |
| 2.33–3.25 " | | | | | | 0.671 | |
| 6.00 p.m. | 15.62 | 5.845 | 2.67 | 1.95 | 0.730 | | |
| Aug. 15, 10.00 a.m. | 29.40 | 9.191 | 3.20 | 1.84 | 0.574 | | 500 gm. beef heart at 10.00 a.m. 18 Rochester units E-29-30-S subtaneously at 12.00 noon. |
| Total for 24 hrs. | 45.02 | | | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 12.27–1.28 p.m. | | | | | | 0.745 | |
| 1.28–2.29 " | | | | | | 0.715 | (Some activity.) |
| 2.29–3.24 " | | | | | | 0.726 | " " |
| 6.20 p.m. | 14.08 | 7.280 | 1.93 | 1.69 | 0.874 | | |
| Aug. 16, 10.00 a.m. | 29.00 | 7.707 | 3.76 | 1.85 | 0.492 | | 500 gm. beef heart at 10.00 a.m. 40 Rochester units stearic acid-coated tablets at 9.00, 9.30, and 10.00 a.m. (See note.) |
| Total for 24 hrs. | 43.08 | | | | | | |
| 12.11–1.15 p.m. | | | | | | 0.687 | |
| 1.15–2.08 " | | | | | | 0.699 | |
| 2.08–2.56 " | | | | | | 0.695 | |
| 6.00 p.m. | 21.60 | 5.740 | 3.76 | 2.70 | 0.717 | | |
| Aug. 17, 10.00 a.m. | 33.35 | 9.632 | 3.46 | 2.08 | 0.602 | | 500 gm. beef heart at 10.00 a.m. |
| Total for 24 hrs. | 54.95 | | | | | | |
| 6.00 p.m. | 21.10 | 6.104 | 3.45 | 2.64 | 0.763 | | |
| Aug. 18, 10.00 a.m. | 33.35 | 9.772 | 3.41 | 2.08 | 0.611 | | |
| Total for 24 hrs. | 54.45 | | | | | | |

The tablets given had the following composition:

| | gm. | |
|---|---|---|
| Amyl ppt. | 0.633 | (100 Rochester units) |
| Salt ppt. | 1.230 | ( 10 " " ) |
| Blood serum (dried) | 1.500 | |
| Malic acid | 0.500 | |
| | 3.863 | = 110 Rochester units. |

Four tablets, weighing 1.4 gm. (total), were coated with stearic acid and given 1 at 9.00, 1 at 9.30, and 2 at 10.00 a.m. along with 10.00 a.m. feeding of beef heart on Aug. 16.

and Table X) is taken into account the effect of oral administration is more than one-half as great without making allowance for the sugar which must have come from the protein of blood serum in the first 6 hours. The decrease from the oral administration of salt precipitate together with blood serum the 1st day is 5.8 gm. (49.6 to 43.8) while on the day of subcutaneous administration it is 9.1 gm. (43.8 to 33.7). Most of the effect from subcutaneous administration came the first 6 hours, but on account of the early excretion of sugar from the blood proteins, the effect after oral administration is seen equally later in the day. On the 2nd day of insulin and blood serum the early effect is masked still more.

*Dog 12.*—Female Boston bull terrier weighing 13.6 kilos. Dog was fed 500 gm. of lean beef heart each day. Phlorhizin given on August 10, 1.5 gm. per day rubbed up with olive oil. Animal remained in splendid condition throughout the experiment. Collection was begun on August 12, the 3rd day of phlorhizin, at 12.00 noon. Respiration experiment proved the dog incapable of oxidizing glucose. Urine at 8.00 p.m. showed D:N ratio of 3.66 (Table XI). The next day the respiration experiment again indicated complete inability to oxidize sugar, the one R.Q. above the diabetic level being due to error in the determination on account of activity of the animal while in the respiration apparatus. Satisfactory controls having thus been established, insulin was given subcutaneously on August 14, 2 hours after a meal of meat. There was an immediate effect on the R.Q. in the 1st hour determined from 12.27 to 1.30 but the quotient returned to diabetic level in the next 2 hours. The effect on the excretion of sugar is evident by the lower peak in the dextrose-per-hour curve at the 6 hour interval (Chart 6). On the next day the dosage of insulin was increased to 18 rabbit units but the effect upon the R.Q. remained small, the highest quotient falling in the first period, as before. The effect, however, is somewhat more sustained than on the previous day when the smaller dose was given. This time there was a marked effect on the excretion of sugar, indicated by the depression in the curve (Chart 6) at the 6 hour interval. There was also a much greater elimination of nitrogen the first 6 hours which is quite exceptional.

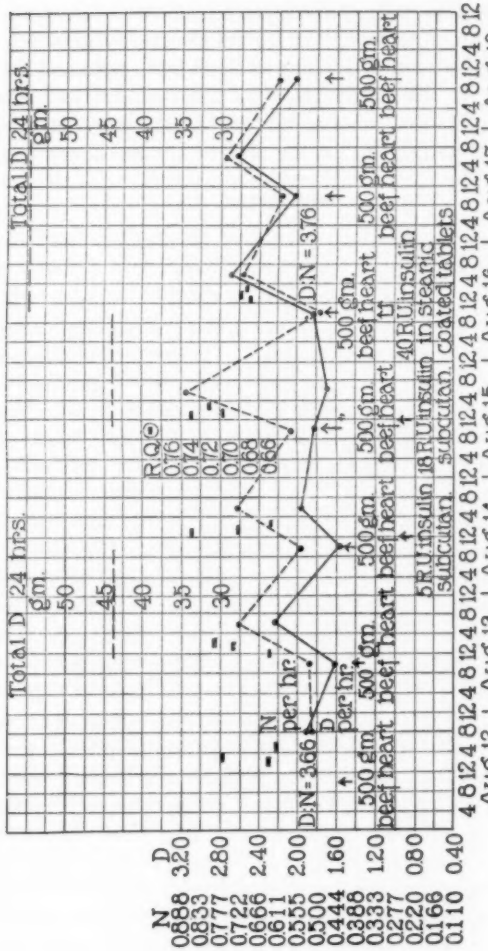
Total D 24 hrs.
gm.
Total D 24 hrs.
gm.
R.Q.
D:N = 3.66
D:N = 3.76
N per hr.
D per hr.
500 gm. beef heart
40 R.U. insulin
5 R.U. insulin
8 R.U. insulin in stearic subcutan. coated tablets
Aug. 12 | Aug. 13 | Aug. 14 | Aug. 15 | Aug. 16 | Aug. 17 | Aug. 18

Chart 6. Dog 12. Fed on meat.

Following this experiment, the D:N ratio on the morning of the 16th was at the diabetic level.

The stearic acid coating was again tried on this day, the tablets being made up as indicated in the foot-note to Table XI. The respiration experiment beginning at 12.11 and continued nearly to 3.00 showed no rise of quotient and the curves of nitrogen and dextrose elimination likewise indicated no effect of absorbed insulin. The control day on the 17th terminates the experiment.

## DISCUSSION.

The curves representing excretion of dextrose and nitrogen on the hourly basis, in all the charts except Chart 1, are so spaced with reference to the ordinates that the two coincide when the D:N ratio is 3.60. Also in Chart 1 the spacing approaches this numerical arrangement very closely. Inspection of these curves in the light of the accompanying respiratory quotients reveals the fact that inability to oxidize glucose does not depend upon the attainment of any given D:N ratio (*e.g.* July 15, Chart 4); but that when the ratio approaches 3.6 the R.Q. is always close to 0.69 (average for the day).

It is indicated therefore that in phlorhizin diabetes, in contrast with pancreatic diabetes, some other factor than non-combustion determines the excretion of sugar. The original observation of Stiles and Lusk (14) that glucose administered to the phlorhizinized dog is eliminated quantitatively only when given in small amounts, supported by the more recent observations of Sansum and Woodyatt (15) and of Nash and Benedict (16) that large quantities are only partially eliminated, bear witness to the same idea; namely, that carbohydrate metabolism is not completely in abeyance, but that its excretion is governed in part by a renal impairment. The low blood sugar characteristic of phlorhizin intoxication is generally held to support this view. In evaluating the effect of insulin, therefore, it is necessary to consider the evidence for combustion and for renal effect separately. An alteration in the rate of excretion or a change in the D:N ratio might be accounted for by combustion or by improvement or further impairment of the renal mechanism.

The regularity of the curves of dextrose and nitrogen excretion

both in the case of otherwise fasting dogs fed glucose and in the case of dogs fed meat (Dog 12) is striking; and the depression in the sugar curve accompanied as it invariably is, if considerable, by a rise in the respiratory quotient (at least in the early respiration periods) caused by insulin makes the demonstration of this effect perfectly convincing. *Sugar is held back which otherwise would be excreted and a part of it is oxidized.* The effect is greater from subcutaneous than from oral administration, as would be expected, but is perfectly evident from both. With Dog 6 we saw that 0.35 gm. glucose were retained, and utilized somehow, for each clinical unit of insulin given subcutaneously. With Dog 8 on June 28, it was possible to account for at least 0.48 gm. for each unit and in this instance there was no doubt about combustion, for the R.Q. continued well above the diabetic level at least 3 hours. Likewise on the 23rd with the same dog it is possible to account for 0.28 gm. of glucose per unit of insulin introduced subcutaneously, but in this instance the rise in R.Q. lasted only 1 hour.

We were not fortunate enough to secure clear-cut data from which the exact amount of combustion could be calculated after oral administration. There is evidence however of another sort for improvement in carbohydrate metabolism. Examining the curves of nitrogen excretion minutely it is evident that there is in the five fasting dogs, Nos. 7 to 11, a very regular behavior with reference to the influence of glucose ingested. Dog 11 furnishes perhaps the best single example (Chart 5). Starting at the moment of glucose ingestion on July 28, there is, during the first 6 hours, a gentle decline in the excretion succeeded in the next 6 hour period by a similiar decline. The absence, or nearly total absence, of effect of the insulin given by mouth on July 29 to this dog is inferred quite as much by the similarity of the nitrogen curve of this day to those of July 28 and August 3 as by the regularity of the sugar curve. When insulin is really effective, however, as with Dog 9 (Chart 4), the primary fall in nitrogen is more precipitous than when insulin is not given and continues at the same rate (Dog 9) or at a greater rate (Dog 8, June 23 and 27) in the second 6 hour period. Here we have a visualization of the sparing effect of glucose when it is not burned contrasted with the sparing effect when it is burned. The first

portion spared corresponds to A. I. Ringer's (17) "dextrose nitrogen" and the second to his "complementary nitrogen."[2] Now subsequent to the oral administration of insulin (*e.g.* Chart 5, July 30) we have the same precipitous drop in the nitrogen as after its introduction under the skin (August 2), though it is sometimes delayed until the second 6 hour period (*e.g.* Chart 5, July 31, and Chart 2, June 11). The later and more prolonged rise in the respiratory quotient in the latter case shows that the tablet was not disintegrated in the intestine immediately; hence the insulin could not become effective so soon as after subcutaneous use. At times the tablets were entirely too resistant to the solvent action of the intestinal juice (*e.g.* Chart 5, July 29, and Chart 3, June 28). In other experiments the effect was positive though small (Chart 3, June 25, and Chart 5, July 30 and 31).

Turning now to the renal factor we have the following observations to report in possible support of the idea that insulin may affect the kidney tubules in a manner antagonistic to that of phlorhizin itself. Dog 4 also Dog 3 with oleic acid exhibited an effect on the blood sugar which is very puzzling (Table III). Large doses of insulin in cod liver oil emulsion had been given on May 4, at 12.45 and 4.05 p.m. The rate of dextrose excretion fell off more rapidly than on the control day, May 5, and *pari passu* the blood sugar rose. In fact the blood sugar was still higher at 1.40 p.m. next day. If sugar escapes through the kidney, as Zuntz (18) was the first to suggest, because the attractive power of the kidney for sugar is increased or, as we should say today, because the convoluted tubule is altered by the phlorhizin so that the sugar which filters through the glomerulus cannot be reabsorbed, any alleviation of this condition would cause retention and consequently a higher blood sugar. No respiration experiment was done on this day; hence we cannot be sure whether any sugar burned. Taking

[2] There seems to be no close relationship between the size of the "dextrose" and "complementary" fractions and the exact level of the D:N ratio. Dogs 7 and 9 had high ratios (curves not coinciding often) while Dogs 8 and 11 had low ratios (nitrogen curve often higher than the dextrose curve) and yet the nitrogen behavior is quite similar in the two types. Dogs 7 and 9 each received two doses of phlorhizin daily, while Dogs 8, 10, and 11 received only one dose, but Reilly, Nolan, and Lusk (8) long ago proved that the D:N ratio is not dependent upon the dosage of the drug so long as it is adequate to produce a prolific glycosuria.

the curve of excretion alone the evidence was not considered significant (p. 735).

Dog 12 also exhibits a peculiar effect (see Chart 6). The dog was fed 500 gm. beef heart daily at 10.00 a.m. On the 14th and 15th of August insulin was introduced subcutaneously, first in a small dose, next day in a very large dose. The effect, the second time, is seen primarily in the depression of the sugar curve, and consequent drop in the D:N succeeded by a rise in the sugar excretion restoring the D:N or even raising it next morning. The curious thing however is the enormous rise in nitrogen excretion, coincident with the depression of the sugar curve. Could this be the consequence of a temporary restoration of the urea-secreting function of the tubules together with a restoration of the power to reabsorb glucose? Nash and Benedict (16) have shown that the power of the kidney to eliminate urea is impaired by phlorhizin even more than the power to reabsorb glucose. There is just a suggestion of the same effect in Dog 1 (Table I) where a soap tablet was given by mouth. This is the only other dog of this series which received meat regularly throughout the experiment. Why giving meat should make any such difference from all the other dogs is not clear, except on a quantitative basis.

In Dog 6 we have quite a different phenomenon. The dog died in coma following complete suppression of the urine and it is possible that the effects produced by the tablets on June 2 (Chart 1) were due, as suggested (p. 745) in part to further injury to an already damaged kidney. Did insulin play any part in the injury, or should it be ascribed to phlorhizin alone or to the malic acid contained in the tablet? Nothing like this syndrome was seen in any other dog. The effect of malic acid is being studied.

The observation just recited is the only support we can offer to the idea of retention under the influence of insulin adopted by Colwell (19). This author is much impressed with the later excretion of sugar temporarily held back when insulin is given both in his own and in Nash's (20) and M. Ringer's experiments. Cori (21) has shown, however, that in phlorhizinized rabbits at least insulin causes a marked increase in glycogen formation in the liver, and as soon as the insulin wears off, the glycogen diminishes under the influence of phlorhizin, and returns to the urine as sugar. In the absence of any positive evidence of kidney injury by insulin

in depancreatized animals, or in human diabetics, it would seem that the glycogen synthesis is the more rational explanation to adopt. One can only wonder how the evidence for combustion in Ringer's paper escaped Colwell's notice. Do not increased combustion and glycogen formation in the normal animal after a heavy meal of carbohydrate run hand in hand? If insulin should counteract the effect of phlorhizin, what would be the evidence? (1) Diminished excretion of sugar. (2) Rise in the R.Q. (3) Sparing of protein. (4) Glycogen formation. The evidence seems to be complete.

What light does this evidence throw upon the mechanism of phlorhizin diabetes? Ringer hazarded the rather hasty opinion that phlorhizin acts upon the pancreas, stopping the production of insulin. But this view, at least so far as a complete inhibition of the insulin production is concerned, has been disposed of by Nash and Benedict (22) and by Cori (23) who have demonstrated the presence of insulin in the pancreas of phlorhizinized dogs and cats, in nearly if not quite normal amounts. Nash and Benedict in their earlier paper postulated the formation of a stable union of the drug with the carbohydrate "receptors" of the cells so that combustion becomes impossible. If this were the correct explanation, it would be necessary to suppose either (1) that insulin breaks this bond and releases the "receptors" of muscle cells especially for combustion and of liver cells especially for glycogen formation; or (2) that it directly antagonizes phlorhizin, possibly by union with the glucoside itself.

There is no evidence that we are aware of supporting the idea of sugar receptors or that insulin and phlorhizin unite thus neutralizing each other. There is evidence, however, that insulin is inactivated by phlorhizin in much the same way that it is by glucose (24).

This evidence has been obtained by Mr. Nathan Francis in this Laboratory by noting first that when phlorhizin and insulin were injected into the same site there was much less effect of the insulin. The observation has been confirmed repeatedly and it appears to make no difference whether the two substances are mixed together previous to injection or not. Great variations in the effects of insulin in phlorhizin diabetes have been recorded in this paper. Some of them probably are to be explained by

inattention to the matter of proximity of injections; others, no doubt, to the differences in the quantity of phlorhizin circulating at the time of insulin injection.

We suggest therefore on the basis not of evidence presented in this paper but more especially upon the evidence produced in cooperation with Mr. Francis and Mr. Allen that phlorhizin diabetes is caused in part by inactivation of insulin after it has reached the circulation or the tissues. Restoration of function by insulin then would be accounted for by reestablishment of insulin dominance. The renal effect of phlorhizin probably calls for another explanation.

The mechanism of the inactivation will be studied further, but we anticipate it will be explained by hydrolysis of phlorhizin in the tissues thus setting free the reducing group of the glucoside sugar and that the chemical reaction will be similar to the antidoting effect of sugars (25).

## SUMMARY.

The influence of insulin introduced subcutaneously and orally into phlorhizinized dogs has been studied in twelve subjects. Phlorhizin was administered by the Coolen method (in olive oil) once or twice daily. All urine periods were separated by catheter. Sugar was determined by Benedict's method, and nitrogen by the Kjeldahl-Gunning method. Blood sugars were taken only occasionally and were determined by the Folin-Wu or by the Hagedorn-Jensen method. The respiratory metabolism was followed in seven subjects by means of a closed circuit apparatus, including a chamber, of the Benedict type. The insulin was made in this laboratory by the rapid method (7), *i.e.* amyl alcohol precipitation, and for oral administration was made into enteric coated tablets or pills from already mixed material supplied to them by the Sharpe and Dohme laboratories in Baltimore, or was combined as desired with various substances which might be supposed to delay the destructive action of trypsin, at once in the laboratory. Two animals only of the twelve were fed on a regular diet of lean beef heart. Dogs 6 to 11 inclusive were given glucose (Merck's C.P. dextrose) in exact amounts every day by stomach tube. The effects of insulin on the hourly excretion, as well as total for 24

hours, of dextrose and nitrogen were studied simultaneously with the respiratory quotient.

## CONCLUSIONS.

1. Insulin introduced subcutaneously into dogs completely under the influence of phlorhizin acts immediately to cause a reduced rate of excretion of sugar, and an increase in the respiratory quotient. When the D : N ratios are below 3.65 there is a greater effect on the R.Q. than when they are above this value.

2. When glucose is given the rate of excretion of nitrogen is reduced (protein is spared) but when glucose and insulin both are given it is reduced still more. These two fractions represent the "dextrose" and "complementary" nitrogen of A. I. Ringer.

3. Insulin administered orally in enteric coated tablets combined with malic acid, sodium oleate, and an amino acid which liberates HCl, also in solution in blood serum, has given positive though very moderate results on the reduction of urine sugar, increase in the R.Q., and sparing of protein. The effect is much less in amount, especially as regards excretion in the early hours, than from subcutaneous administration.

4. The sugar which disappears from the urine and does not reappear may amount to from 0.28 to 0.48 gm. per clinical unit (one-third "Rochester unit") of insulin administered subcutaneously. No exact estimate could be made after oral administration but, as expected, it is very much less than after subcutaneous use.

5. Some of the sugar which disappears from the urine may reappear when the insulin effect wears off because as M. Ringer has suggested, and as Cori has shown for the rabbit, it may be stored temporarily as glycogen.

6. Evidence that phlorhizin inactivates insulin is referred to and the hypothesis is presented that phlorhizin produces diabetes in part by rendering inert the insulin already in the tissues. Insulin injected in sufficient amount is restored to dominance.

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# A METHOD FOR THE DETERMINATION OF THE ENERGY VALUES OF FOODS AND EXCRETA.

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The determination of the energy values, specifically the heat of combustion, of various foods and excreta forms an important part of many investigations in nutrition. The calorimetric bomb is commonly used for these determinations, but its complexity of manipulation and its almost prohibitive cost greatly narrow the opportunities for making such studies. Moreover the calculation of results is time-consuming and usually involves more mathematics than are warranted by the highest limit of accuracy possible in the preparation of the sample from unhomogeneous material. As an outcome of research on the respiration of man and the development of simplified apparatus for measuring the actual oxygen consumption during the processes of oxidation in the body, a principle for the determination of the energy values of foods and feces in the case of both men and animals has been experimentally tested and embodied in an apparatus which is less complicated, less expensive, and which enables simpler calculation of results than does the bomb calorimeter.

The fundamental principle of this apparatus is the direct determination of the volume of oxygen required to burn a known weight of a substance and the computation therefrom of the potential energy of the substance, based upon a series of factors for the calorific value of a liter of oxygen previously established by combustion of similar material in a bomb calorimeter. In a pure substance, such as sucrose, the amount of oxygen involved in the combustion of 1 gm. can readily be computed. Since the heat of combustion is accurately known, the calorific value of each liter of oxygen can be calculated. These relationships for a number of

commonly metabolized compounds have been computed (1) and are given in Table I.

Thus with these pure substances, if the amount of oxygen required to oxidize 1 gm. of substance is known, the heat of combustion can be accurately computed by the use of the factors in the last column of Table I. Since food materials and feces consist for the most part of the substances listed in Table I, particularly of protein, fats, and carbohydrates, it would be possible to compute the energy values of various foods by estimating the proportion of protein, fat, and carbohydrate in the food (based upon

TABLE I.

*Gaseous and Energy Relationship in the Combustion of Some Commonly Metabolized Compounds.*

| Substance. | Oxygen required to oxidize 1 gm. | Produced in the oxidation of 1 gm. | | Calories per liter of oxygen. |
|---|---|---|---|---|
| | | Carbon dioxide. | Heat. | |
| | *cc.* | *cc* | *cals.* | |
| Starch | 829.3 | 829.3 | 4.20 | 5.06 |
| Cane sugar | 785.5 | 785.5 | 3.96 | 5.04 |
| Dextrose | 746.2 | 746.2 | 3.74 | 5.01 |
| Lactic acid | 745.9 | 746.0 | 3.62 | 4.85 |
| Animal fat | 2013.2 | 1431.1 | 9.50 | 4.72 |
| Human " | 1990.8 | 1420.4 | 9.54 | 4.79 |
| Protein | 956.9 | 773.8 | 4.40 | 4.60 |
| Acetone | 1542.9 | 1157.2 | 7.43 | 4.82 |
| $\beta$-Oxybutyric acid | 968.2 | 860.7 | 4.69 | 4.85 |
| Ethyl alcohol | 1459.5 | 972.9 | 7.08 | 4.85 |

already existing food analyses) and applying the established calorific value per gm. of each of these substances. The range in these calorific values is very wide, however,—from 4 to as high as 9 calories—whereas the range in the calorific value of a liter of oxygen is only from 4.6 in the case of protein to 5.0 in the case of carbohydrates. Actually determined calorific values of oxygen in the case of various foods and feedingstuffs and excreta of man and beasts would therefore enable more exact approximations to the true energy values of the substances in question. By comparing the oxygen involved in the combustion of 1 gm. of substance directly with the heat of combustion as determined in the calori-

metric bomb, one can establish such ratios. When the calorific values of oxygen have thus been established, it is only necessary

TABLE II.

*Calorific Values of Oxygen for Various Foods, Feedingstuffs, and Excreta.*

| Substances. | Calories per liter of oxygen. |
|---|---|
| High carbohydrate substances. | |
| Dried skimmed milk | 4.89 |
| Oyster crackers | 4.90 |
| Corn-meal | 4.88 |
| Nut bread | 4.88 |
| Cheese sandwich | 4.95 |
| Chicken " | 4.95 |
| Salmon salad sandwich | 4.98 |
| Club sandwich | 4.93 |
| Doughnut | 4.90 |
| Highly nitrogenous substances. | |
| Glidine (vegetable protein) | 4.67 |
| Ossein | 4.69 |
| Collagen | 4.70 |
| Plasmon | 4.65 |
| Fats. | |
| Olive oil | 4.74 |
| Corn " | 4.71 |
| Cottonseed oil | 4.70 |
| Cod liver oil | 4.70 |
| Goose fat | 4.75 |
| Butter | 4.62 |
| Mixed foods. | |
| Beef stew | 4.84 |
| Mince pie | 4.97 |
| Animal foods. | |
| Hay, Specimen I | 4.80 |
| " " II | 4.86 |
| Cottonseed meal | 4.66 |
| Linseed meal | 4.76 |
| Gluten " | 4.85 |
| Excreta. | |
| Human feces | 4.97 |
| Steer " | 4.84 |

to measure directly the oxygen involved in the combustion of a food and, taking into consideration the general character of the substance being burned, to apply the heat factor previously de-

termined from the actual combustion of similar materials. A rather extensive series of such factors is presented in this paper (see Table II), together with a description of the method for the direct determination of the oxygen absorbed in the combustion.

This procedure obviously requires a combustion chamber, a method of ignition, and the control of the combustion, in order to insure complete conversion of all the carbon and hydrogen in the sample to carbon dioxide and water and the liberation of nitrogen in the free form in the case of nitrogenous materials. A known weight of substance is burned in a confined volume of nearly pure oxygen, with provision for the rapid absorption by soda-lime of the carbon dioxide produced and the return of the purified oxygen to the combustion chamber. The contraction in volume due to the absorption of oxygen in the process of combustion may be measured by a small expansion chamber, the top of which is brought back to its original level by introducing a measured volume of air or oxygen after the combustion ceases, or it may be measured by noting quantitatively on a calibrated spirometer bell the decrease in the apparent volume of air in the entire apparatus.

The application of this principle in the estimation of the heating value of fuels has already been described elsewhere (2). The apparatus perfected for this purpose has been styled the "oxycalorimeter," since it measures the heat indirectly by reference to the oxygen consumption. This most precise form recognizes the necessity for great accuracy in the estimation of the energy value of fuels, because of their high economic value and financial importance. Hence in the measurement of the contraction in volume of oxygen in this special form of apparatus every effort is made to secure standard, reproducible conditions of temperature, pressure, and particularly humidity.

When the energy values of foods and excretory products are to be studied, however, a simpler device with a sufficiently high degree of accuracy to meet all conditions in problems in nutrition may be employed. Thus, any one of the numerous simple forms of closed circuit respiration apparatus may readily be adapted to the direct determination of the oxygen consumption involved in the combustion of a known weight of previously dried food material, and this type of apparatus is especially recommended for use in

hospitals, dietetic laboratories, and particularly those institutions already possessing some simple form of respiration apparatus. The fundamental principle of the apparatus remains the same. Since, however, the error in the sampling of foods and excreta may be as high as 5 per cent or more, special provisions for the control of temperature, pressure, and particularly humidity are entirely unnecessary and, indeed, not justified.

The combustion chamber and method of ignition are common to all forms of the oxy-calorimeter, but several methods for the measurement of the oxygen absorption are available.

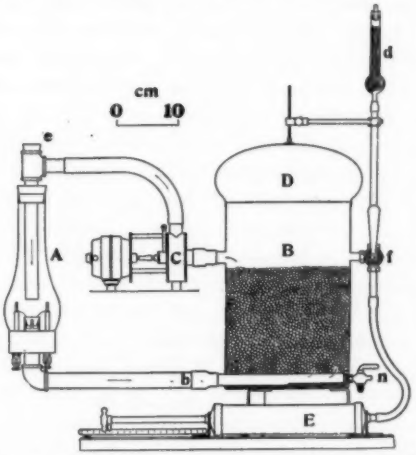


FIG. 1. The student respiration apparatus employed as an oxy-calorimeter for determining the energy values of foods, feedingstuffs, and excreta.

*Use of the Student Respiration Apparatus as an Oxy-Calorimeter.*

Of the various forms of apparatus for the measurement of the oxygen consumption of humans the simplest, accurate form with which we are familiar is that described by the Nutrition Laboratory as a "student form of respiration apparatus" (3). This apparatus, when combined with a combustion chamber and a motor-blower device for controlling the ventilation of the system, serves equally well for measuring the oxygen absorption during the combustion of a sample of food or excreta. The details of the ap-

paratus, as thus set up, are shown in Fig. 1. A main feature of the student respiration apparatus is a copper can, *B*, two-thirds filled with soda-lime and covered with a pure gum, rubber bathing cap, *D*. When this apparatus is used as an oxy-calorimeter, the usual inspiratory and expiratory valves are removed and the apparatus is connected with a combustion chamber, *A*, and a motor-blower unit, *C*.

*Combustion Chamber.*—The combustion chamber consists of a water-sealed, glass vessel, a lamp chimney of standard size, preferably of a glass such as Pyrex with a low coefficient of expansion. The lower end of the chimney is sealed in water in a brass cup, through which is passed a $\frac{3}{4}$ inch standard brass pipe and two rods of nickel or nickel alloy, fitted with electric binding posts at the bottom, one of which is insulated from the brass base by hard rubber washers. Into the upper part of the brass pipe wide slots have been sawed so as to make three prongs into which a nickel crucible is easily placed, thus providing sufficient space to allow the free passage of oxygen between the bottom of the crucible and the opening in the pipe. The upright $\frac{3}{4}$ inch pipe in the base is screwed into a standard $\frac{3}{4}$ inch elbow. In the top of the lamp chimney is placed a one-hole rubber stopper carrying a standard $\frac{1}{2}$ inch brass pipe, 15 mm. in internal diameter. This is fitted to a tee at the top provided with a rubber stopper, *e*, and a side connection of $\frac{1}{2}$ inch standard pipe, which is connected by a rubber hose to the blower, *C*. At no point in the circuit is there an increased or decreased pressure sufficient to cause the water to leave the water seal. This seal does away with all possibilities of leaks at this point.

*Rotary Blower.*—The circulation of the air in the apparatus should be not far from 30 liters per minute, and a rotary blower of good construction is essential. In this combustion system a simple rotary air impeller is sufficient. A very satisfactory simple blower[1] connects directly with a 110 volt motor, and the length of bearing through the housing of the blower and the lubrication are such as to insure absence of leaks. The rate of speed may be controlled by a simple resistance in the line. The actual dis-

[1] This blower, and indeed the entire apparatus, can be obtained from W. E. Collins, 584 Huntington Avenue, Boston, Mass.

charge of oxygen to the combustion chamber may further be regulated by placing a large screw pinch-cock on the rubber tube connecting the blower and the lamp chimney.

*Ignition.*—The electrical method of ignition, commonly employed in the calorimetric bomb, is likewise here used. From the diagram in Fig. 2 it is seen that by closing the electric circuit with a key or push-button a 110 volt current is caused to pass through a heating resistance of about 20 ohms, through an insulated nickel alloy post in the base of the combustion chamber, and across a fine iron wire (75 mm. long and No. 33 B and S gauge, *i.e.*, 0.18 mm. in diameter) which is attached between the two

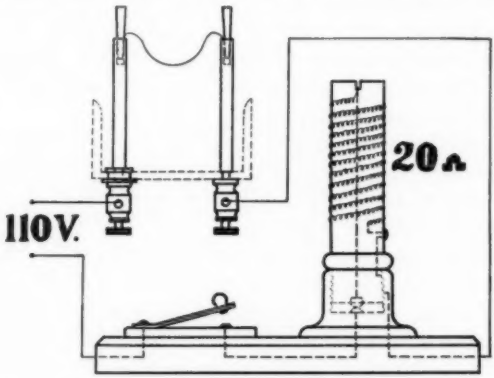


FIG. 2. Details of electric circuit used in ignition of substance in the combustion chamber of the oxy-calorimeter.

nickel alloy posts. On closing the circuit by the key, the wire is burned by the 5 amperes of current and the circuit is automatically broken. On releasing the key or push-button there is no arcing. The iron wire used for ignition is simply held in place by inserting the two ends in holes drilled in the ends of the two nickel alloy rods, and then putting bits of alloy rod, slightly smaller in diameter and tapered, in the tops of these holes to crowd down and hold the wire in place. The holes are 4 mm. in diameter and 10 or 15 mm. deep. Any resistance of 20 ohms, capable of withstanding 5 amperes, may be used. A common, inexpensive heating resistance employed in small, portable, electric stoves or electric

heaters has been found very satisfactory. This resistance screws into an ordinary lamp base. The oxygen required to burn the iron wire, computed to be about 5 cc., should be taken into account in the final calculation of results.

*Soda-Lime.*—With the student form of respiration apparatus the quality of the soda-lime is entirely immaterial, so long as it absorbs carbon dioxide. We have commonly used either some of the patent soda-limes on the market or a modified form of Haldane soda-lime.

*Preparation of Samples.*—As in any bomb calorimeter, the material must be sufficiently dry to burn readily, and preliminary desiccation is therefore generally necessary. Approximately 1 or 2 gm. of the air-dry material, accurately weighed in a nickel crucible, are ample for the combustion. Usually sufficient material is employed to insure that the apparent volume of oxygen absorbed is between 2 and 3 liters.

*Crucible.*—The sample is laid loosely in a pure nickel crucible, spun or stamped out of sheet nickel, 0.5 mm. thick. These crucibles are 22 mm. deep, with a diameter at the top of 32 mm., and have a total capacity of 9 cc.

*Oxygen-Measuring Device.*—In the arrangement as shown in Fig. 1 the soda-lime for the absorption of the carbon dioxide is inside of the can, and the contraction in volume, or the oxygen absorbed, is measured by noting the amount of *air* which must be admitted to the respiration apparatus to bring the index button on top of the flexible rubber bathing cap, *D*, back to its initial point at the beginning of the test; *i.e.*, in contact with the index needle. This measurement is made in a most simple manner by means of the pump, *E*, which is an automobile grease pump slightly modified (4). At the beginning of the experiment the system is filled by admitting pure oxygen through the pet-cock, *n*. At the start the bathing cap, *D*, is well depressed into the can, *B*, and the stopper, *e*, at the top of the combustion chamber is removed. On replacing the stopper, the bathing cap is distended gradually by the further introduction of oxygen, until the index button *just touches the needle*. The motor-blower is then started and the substance is ignited. At the bottom of the combustion chamber is an elbow with a rather long pipe, *b*, leading to the pipe in the base of the can, *B*. It is essential that the pipe, *b*,

should telescope well over the pipe in the bottom of the can, in order to have a "metal to metal" joint, the final closure being made by a short piece of rubber tubing. With this type of closure the heat from the combustion will never be sufficient to damage the rubber, much less ignite it. Ordinarily no cooling is necessary, although one can, if necessary, easily lay a wet cloth on the pipe, *b*.

*Standardization by the Combustion of Sucrose.*

A description of the use of the student respiration apparatus in the combustion of pure sucrose, which serves as an excellent substance for standardization tests, will illustrate the general method of employment in burning any foods or excreta. From 2 to 2.5 gm. of pure sucrose are put in the nickel crucible, which is then placed inside the combustion chamber. The ignition wire is attached to the two upright nickel alloy rods, and a small pinch of powdered pumice stone is sprinkled about the wire where it rests on the sucrose. After the lamp chimney has been replaced in the water seal, the whole system is filled with oxygen, the rubber stopper is inserted in the top of the lamp chimney, and the bathing cap is brought to the position of contact with the index needle. The motor is then started and the substance is ignited. The brilliancy of combustion is such that colored glasses should be worn by the operator.

The small amount of powdered pumice stone on the surface of the sugar makes the ignition certain. The $\frac{1}{2}$ inch pipe conducting oxygen into the combustion chamber restricts the discharge so that the oxygen impinges directly upon the surface of the burning sugar. This tends to control the combustion, prevents the substance from frothing unduly, and prevents particles of charred sugar from being blown out of the crucible. Usually the combustion is complete, leaving no residue except the ash from the pumice stone, at the end of 1 or 2 minutes.

After the combustion has ceased and the combustion chamber has returned to its original temperature and the ventilation is stopped, the bathing cap is considerably depressed, corresponding to the decrease in volume of the gas inside of the system, which represents the apparent volume of oxygen consumed. To determine the oxygen consumed the pump, *E*, is used. This pump is

connected directly with the 3-way valve, *f*, which communicates through a pipe with the calcium chloride tube, *d*, at the top, and likewise communicates with the can, *B*. With the valve in the position shown in the diagram, the piston of the pump is drawn out and the pump becomes filled with *dry air*, dried by the calcium chloride in *d*, and at the end of the stroke this air is at the prevailing atmospheric pressure. The temperature is obtained from a thermometer placed with its bulb resting on the barrel of the pump or placed in a small tee in the rubber hose connecting the pump and the 3-way valve. The operator's hands must never touch the barrel of the pump. By turning the valve, *f*, 90°, this pumpful of dry air may be discharged into the can, B, thus refilling and finally distending the rubber cap, *D*. With the type of pump commonly furnished with this apparatus each complete stroke corresponds to not far from 350 cc., or exactly 1.872 cc. per mm. length of stroke. By repeating this operation successive pumpfuls of dry air are discharged into the can until, at the end, a fraction of a pump stroke will be necessary to bring the index button on top of the bag just in contact with the vertical needle. From the number of full pump strokes, plus the actual length of the fractional pump stroke at the end, the total amount of *dry air* (at room temperature and the prevailing atmospheric pressure) required to replace the oxygen absorbed by the weight of substance burned is easily calculated.

This particular form of oxy-calorimeter was controlled by numerous combustions of pure sucrose, lactose, benzoic and salicylic acids, and typical nitrogen-containing substances such as uric and hippuric acids, and gave results in excellent agreement with the theoretical amount of oxygen required in the combustion of 1 gm. of the substance.

*Calculation of Results of a Combustion of Salmon Salad Sandwich, Using the Student Form of Apparatus.*

A typical calculation of the heat of combustion of a sample of mixed food is that for a salmon salad sandwich. The apparent volume of oxygen absorbed during the complete combustion of the sample was measured when almost six full strokes of the air pump were needed to bring the bag back to its original position. The apparent volume of air introduced is computed from the internal

diameter of the pump and the length and number of strokes. There is no aqueous tension to be considered, for the air drawn into the pump is first dried by passing it over calcium chloride. The apparent volume may then be reduced to 0°C. and 760 mm. by the formula

$$V \times \frac{273}{273 + t} \times \frac{p}{760}$$

in which $t$ is the temperature of the pump barrel, and $p$ the observed barometer.

By using the tables prepared by Carpenter (5) for reducing volumes of dry air to 0°C. and 760 mm. the formula can be more simply stated:

$$V = \frac{(l \times K \times m) - 5}{W}$$

in which $l$ is the total length of strokes through which the pump traveled, $K$ is the constant apparent volume of a mm. stroke of the pump, $m$ is the reduction factor at $t$ and $p$, 5 represents the correction for the cc. of oxygen used in the ignition of the wire, and $W$ is the weight of the dried sample of salmon salad sandwich.

In this particular case $l$ represents five full strokes of the pump, each of 185.5 mm., plus a fraction of a stroke which was 180 mm. long. The total length of strokes was therefore (5 × 185.5 mm.) + 180 mm. or 1107.5 mm. $K$ equals 1.872 cc. per mm. length of stroke. The temperature, $t$, was 26.5°C., and the pressure, $p$, was 756 mm., making the reduction factor, $m$, equal 0.907. The correction for ignition of the wire is 5 cc. The sample burned weighed 1.9932 gm. The formula therefore becomes

$$V = \frac{(1107.5 \times 1.872 \times 0.907) - 5}{1.9932} = 941$$

The nitrogen in the sample was found to be 3.1 per cent by weight, or 25 cc. per gm. of substance. This volume of nitrogen took the place of oxygen absorbed and should therefore be added to the amount of oxygen measured per gm.; *i.e.*, 941 + 25 = 966 cc. The last figure represents the volume of oxygen required to burn completely 1 gm. of the salmon salad sandwich.

Although no proximate analysis of the material was made, one

can readily assume that it was made up in large part of carbohydrate and hence the calorific value of oxygen would probably be somewhat less than that for pure starch, *i.e.*, 5.06.

In this case and in the case of all the substances burned in connection with this paper direct estimations of the heat of combustion by a standardized bomb calorimeter were made. According to the combustion in the bomb calorimeter, each gm. of the sample of salmon salad sandwich had an energy value of 4826 calories. The calorific equivalent of 1 liter of oxygen in the case of this material therefore is 5.00 calories (4826 ÷ 966).

*Use of Standard Respiration Apparatus of the Closed Circuit Type as Oxy-Calorimeters.*

Numerous laboratories in the United States possess one of the many types of respiration apparatus using some form of spirometer for measuring the oxygen consumption of humans. In such laboratories, obviously, it is extremely simple to attach the combustion chamber, *A*, and the motor blower, *C*, to the respiration apparatus and with equal facility obtain the data with regard to the oxygen consumption per gm. of substance burned. Such respiration apparatus is usually provided either with respiratory valves or with a mechanical device for circulating the air. The soda-lime for absorbing carbon dioxide is generally inside of the spirometer. In some of the Nutrition Laboratory's earlier forms of apparatus the soda-lime was placed in a bottle outside. By far the greater number now have the soda-lime inside of the spirometer. The attachment of the combustion chamber to this type of apparatus is extremely simple. In the case of those apparatus having the blower inside, it is necessary to remove the blower and attach the combustion chamber and external rotary blower to the two openings in the bottom of the spirometer in such a manner that the air leaving the combustion chamber (being rich in carbon dioxide) enters the base of the soda-lime can and is purified during its passage through it. Some spirometers have two separate elbows at the bottom; in others there is one casting or fitting combining both exit and entrance.

The use of this latter type of spirometer is pictured in Fig. 3. The combustion chamber, *A*, and motor-blower unit, *C*, are con-

nected with the fittings at the bottom of the spirometer. The air is conducted from the combustion chamber through the pipe, *b*, with its "metal to metal" connections, is forced upwards through the soda-lime in the can, *B*, passes into the spirometer bell, *D*, and then down around the soda-lime can and out through the fitting connecting directly with the blower, *C*. The air delivered to the combustion chamber, therefore, is always free from carbon dioxide. The contraction of the spirometer bell, *D*, is noted by a pointer attached to a counterweight traveling over a mm. scale. The

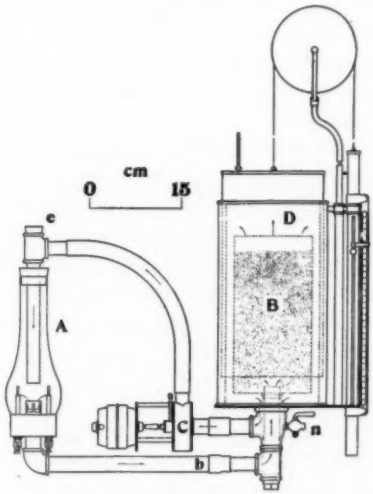


FIG. 3. Another type of simplified oxy-calorimeter for determining the energy values of foods, feedingstuffs, and excreta.

humidity of the air inside the bell is determined by the moisture content of the soda-lime. The contraction of air, which represents the apparent volume of oxygen used, is in ordinary respiration experiments calculated by rather empirical formulas, based on a large number of actual control tests (6).

This apparatus, as pictured in Fig. 3, was likewise controlled by several combustions of pure sucrose, and most satisfactory results were obtained. Its use in the actual combustion of a food

is exemplified by the following typical calculation, based upon the combustion of a sample of doughnut.

*Calculation of Results of a Combustion of Doughnut, Using the Benedict-Collins (7) Portable Respiration Apparatus, with Wilson Soda-Lime inside the Spirometer.*

For this calculation the same formula would be used as given for the student form of apparatus of Benedict and Benedict, namely

$$V = \frac{(l \times K \times m) - 5}{W}$$

In this particular combustion the apparatus used was the Benedict-Collins apparatus (8) similar to the Roth modification (7); *i.e.*, the blower was not inside the bell and the carbon dioxide entered the soda-lime before entering the bell proper. The volume of air in the spirometer is larger than that in the usual Roth modification, however, and the correction for the rise in temperature of the bell (which is applied directly to the spirometer reading) is therefore 1.8 mm. for each degree (Centigrade) change in temperature. Therefore $l$ would equal the corrected fall of the spirometer bell; *i.e.*, the apparent fall plus the temperature correction. $K$ is the bell factor, and $m$ is the reduction factor for temperature and pressure. In finding the value of $m$ in the standard tables published by Carpenter (5) it should be taken into consideration that in this apparatus the air is partially saturated. The reduction factor should therefore be calculated on the basis of dry air, but Roth's correction (7) of minus 2 per cent for saturated air should be applied to the final reduced volume. The subtraction of 5 cc., due to the ignition of the wire, should be made as usual.

In the particular combustion used for illustration, $l$ equals 108.0 mm. plus 7.7 mm. (correction for the rise of 4.3°C. in temperature) or 115.7 mm. The constant, $K$, is 21.32 cc. for this apparatus. The average temperature during the combustion was 22.9°C., and the barometer read 770 mm. The reduction factor, $m$, for this temperature and pressure is 0.935. The weight, $W$, of the sample was 2.0570 gm. The formula thus becomes

$$V = \frac{(115.7 \times 21.32 \times 0.935 \times 0.98) - 5}{2.0570} = 1{,}096$$

The nitrogen content was found to be 1.1 per cent by weight or 9 cc. per gm. of substance. The total amount of oxygen required to oxidize 1 gm. of the doughnut is therefore 1096 plus 9 or 1105 cc. In view of the well known fatty nature of the doughnut a calorific value of oxygen lying somewhere between 5.06 (that for starch) and 4.7 (that for fats) should be used. An average figure of 4.85 would be plausible. Actual determination of the factor was made by combustion of the substance in a bomb calorimeter, in which it was found that each gm. of the sample of doughnut had an energy value of 5331 calories. The calorific equivalent of 1 liter of oxygen in the case of the doughnut, therefore, is 4.82 calories (5331 ÷ 1105).

*Calorific Value of the Oxygen Involved in the Combustion of Various Foods, Feedingstuffs, and Feces.*

The calorific value of oxygen, as found with the oxy-calorimeter and the bomb calorimeter, for pure cane sugar agrees perfectly with the theoretical value. The calorific values of oxygen determined with the oxy-calorimeter and the bomb calorimeter, when burning the pure substances, lactose, benzoic, salicylic, hippuric, and uric acids, have also been found to be in full accord with the theoretical values. Of special moment in connection with this article, however, are the actually determined calorific values of oxygen in the case of various foods and feedingstuffs and the excreta of man and beasts. A number of such substances were burned and the details of the calorific values of oxygen are given in Table II.

From Tables I and II it is seen that the range in the calorific value of oxygen is extremely small, the lowest value being that for protein (4.60) and the highest values those for carbohydrates. But since much of the food of man and practically all of the food of domestic animals is of a high carbohydrate nature, the calorific value of most foods will be not far from that of carbohydrates, that is, *circa* 5.0 calories per liter of oxygen consumed. While there are occasionally some seeming irregularities in the values in Table II, in general the factor is not far from 4.68 for the nitrogen-rich substances such as protein, 4.70 for fats, and nearly 5.0 for the substances of a high carbohydrate nature.

In the determination of the basal metabolism of humans it is

common custom to assume that each liter of oxygen consumed under basal conditions has a calorific value of 4.825. This implies that 12 hours after the last ingestion of food there is a metabolism of mixed carbohydrate and fat. In general the use of this factor, 4.825, would be thoroughly justified in all estimations of the calorific value of foods. Slightly greater refinement may be made, however, by classifying the foods somewhat as in Table II. If a food substance is obviously of a fatty nature, with visible fat, one would be justified in using the factor 4.7. If a composite sample of all the meals during a day were to be taken, it would be thoroughly justifiable to consider that the calorific value of each liter of oxygen required to burn such a sample would be represented by 4.825, while in the case of substances containing a great preponderance of carbohydrate the high value of 5 would be more representative. In any case it is clear that these factors would lie far inside of the limit of accuracy which it is possible to obtain in preparing a sample of mixed food for combustion.

SUMMARY.

A method has been developed and tested in the Nutrition Laboratory for the indirect determination of the energy values of foods, feedingstuffs, and excreta. The fundamental principle involves the direct measurement of the oxygen consumed during the combustion of a known weight of a substance and the computation therefrom of the potential energy of the substance by means of a series of factors for the calorific value of a liter of oxygen previously established with a bomb calorimeter. The apparatus embodying this principle has been styled the "oxy-calorimeter." A brief description is given of the combustion chamber and the device for ignition of the substance, and the adaptation of simple respiration apparatus as oxy-calorimeters is discussed in detail. Illustrations of the calculation of results are given, together with tables listing the calorific values of oxygen for some commonly metabolized compounds and for a number of various foods and excreta. This simple form of oxy-calorimeter is recommended especially for hospitals, dietetic laboratories, and those institutions already possessing one of the numerous simple forms of closed circuit respiration apparatus. Average calorific values of oxygen for use in connection with determinations with the oxy-calorimeter

are suggested as follows: for nitrogen-rich substances 4.68 calories per liter of oxygen; for fats 4.7 calories; for carbohydrate-rich foods 5.0 calories; for a mixed diet of carbohydrates and fats 4.825 calories; and for feces 5.0 calories.

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# THE EXCRETION OF NORMAL URINE SUGAR.*

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Substances are present in the urine of normal individuals which reduce various sugar reagents. These reducing substances are commonly called sugar. Interest in the normal urine sugar was created by Benedict who described methods for its determination and who reported values for the amounts excreted upon different diets. Recent observations indicate that the amount varies from 614 to 1383 mg., with an average of 941 mg., daily (Neuwirth (1)). A certain part of this is not fermented by yeast. The non-fermentable fraction is said to comprise from 51 to 86 per cent of the total. Kast, Croll, and Myers (2) have found an average daily excretion of 960 mg. of total reducing substances. These values refer to the excretion by normal individuals living upon ordinary mixed diets. There is a greater excretion of sugar often observed following meals. This was first observed by Benedict and the name glycuresis was given to this phenomenon by him to distinguish it from glycosuria which signifies a pathological excretion of sugar.

The origin and the factors governing the excretion of normal urine sugar are in dispute. Folin (3) believes that the sugar has no relation to the main carbohydrate metabolism and that it consists of foreign, unusable carbohydrate materials found in grains, vegetables, and fruits; also decomposition products resulting from the preparation of foods. He maintains that glycuresis is independent of the blood sugar level. Benedict (4)

* Presented in abstract before the American Chemical Society, Los Angeles, August 6, 1925.

has severely criticized Folin's interpretation of the experiments upon which these conclusions were based. He believes that the causes leading to the excretion of sugar are usually the same as those leading to an increase in blood sugar, but he questions whether the two phenomena are necessarily always causally related. This investigator reminds us that under ordinary conditions not more than about 50 per cent of the total sugar of normal urine can be glucose, and that usually the glucose cannot exceed 25 per cent of the total. Shaffer (5) doubts whether any of the reducing substances are fermentable by yeast. A recent paper by Höst (6) contains many data on the excretion of normal urine sugar. This author found that mixed food, and bread, especially the coarser types, caused the excretion of larger amounts of sugar. The oral administration of glucose did not result in an increased excretion of sugar. He believes that the normal urine sugar has no relation to the blood sugar and that the sugar in the urine is probably not glucose. His view is practically identical with that of Folin and Berglund. Felsher and Woodyatt (7) report that the intravenous injection of increasing amounts of C.P. glucose into dogs results in the excretion of increasing amounts of sugar. The increase over the fasting level is not marked until an injection rate of slightly under 1 gm. per kilo per hour is reached, when there is a sharp acceleration in the rate of sugar excretion. These workers incline to the view that the increasing excretions of sugar after intravenous injections accompany like changes in the blood sugar concentration. But there is a sharp break at a certain point and not a gradual transition to gross glycosuria as suggested by Benedict, Osterberg, and Neuwirth (8). Wang and Felsher (9) made the interesting observation that an alcoholic extract of commercial granular glucose when added to C.P. glucose caused a marked increase in the excretion of total reducing substances as compared with the effect of ingesting C.P. glucose alone. The extract had, by itself, only a slight action on the blood sugar. Greenwald, Gross, and Samet (10), in a recent paper, record a number of experiments upon man and dog dealing with the origin and nature of the normal urine sugar. An excellent summary of the literature is given. They conclude:

"The sugars excreted in normal urine are made up of difficultly- or non-assimilable carbohydrates and of reducing substances derived from the protein of the food and from endogenous sources. The nature of those in the former group depends upon the diet. This group may include lactose from milk, pentoses from fruits, and caramelized sugars and dextrins. The amounts of all of these are usually small. Upon ordinary diets, at least one-half of the sugars of the urine originate from the protein of the food or from endogenous sources. Apparently, they do not arise from nuclein metabolism. It is suggested that they may be pentoses, which, it is suggested, are always formed from protein and, in pentosuria, are either formed in larger quantities or escape further catabolism.

"The tolerance for glucose is distinctly lowered by a carbohydrate-free diet. The effect appears to be the greater, the larger the part that fat contributes to the metabolism. Protein appears to counteract the effect of fat. . . . .

"In unquestionably normal individuals, upon a mixed or a carbohydrate diet, not containing any large quantities of glucose, the concentration of glucose in the blood probably never reaches this threshold [glucose threshold] and their urines probably never contain glucose."

The above review indicates the uncertainty regarding the nature of normal urine sugar and the factors which govern the amount excreted. We think that all the data bearing on this subject should be published so that a semblance of order may be established.

We have studied the effects of a number of different types of diet and of the ingestion of glucose upon the excretion of normal urine sugar.

*Plan of Experiments and Methods.*

Three healthy young women served as subjects for the experiments. A uniform basal diet was used throughout most of the study. This diet consisted of protein, 74 gm.; fat, 81 gm.; and carbohydrate, 216 gm. The effects of superimposing commercial and pure glucose upon this diet were determined. Diets varying in content of protein but with the same potential glucose formers were also studied. The urines were collected in 2 hour periods beginning at 8 a.m. and ending at 10 p.m. The remainder of the 24 hour period was united. Total reducing substances and the amounts fermentable by yeast were determined by the method of Benedict and Osterberg (11).

TABLE I.

*Excretion of Sugar after Glucose Ingestion.*

| Experimental days. | Sugar. | | | | | Glucose ingested. | | Diet. |
|---|---|---|---|---|---|---|---|---|
| | Total. | Fermentable. | Non-fermentable. | Fermentable. | Non-fermentable. | Amount. | Type. | |
| **Subject B.** | | | | | | | | |
| | *mg.* | *mg.* | *mg.* | *per cent change* | *per cent change* | *gm.* | | |
| 3 | 487 | 174 | 313 | | | 0 | | Protein 74 gm., fat 81 gm., carbohydrate 216 gm. |
| 2 | 448 | 174 | 274 | 0.0 | −12.5 | 75 | Pure. | " " " |
| 2 | 420 | 99 | 321 | −43.1 | +2.6 | 150 | " | " " " |
| 2 | 452 | 122 | 330 | −29.9 | +5.4 | 225 | " | " " " |
| 2 | 499 | 227 | 272 | +30.5 | −13.1 | 300 | " | " " " |
| 2 | 441 | 149 | 292 | | | 0 | | " " " |
| 2 | 465 | 210 | 255 | +40.9 | −12.7 | 175 | Commercial. | " " " |
| 2 | 515 | 249 | 266 | +67.1 | −8.9 | 350 | " | " " " |
| 10 | 489 | 189 | 300 | | | 0 | | " " " |
| **Subject H.** | | | | | | | | |
| 2 | 528 | 185 | 343 | | | 0 | | Protein 74 gm., fat 81 gm., carbohydrate 216 gm. |
| 2 | 512 | 189 | 323 | +2.2 | −5.7 | 75 | Pure. | " " " |
| 2 | 555 | 246 | 309 | +33.0 | −9.9 | 150 | " | " " " |
| 2 | 524 | 224 | 300 | +21.1 | −12.6 | 225 | " | " " " |
| 1 | 603 | 256 | 347 | +38.4 | +1.2 | 300 | " | " " " |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 603 | 256 | 347 | +38.4 | +1.2 | 300 | " | " | " | " |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 404 | 164 | 240 | | | 0 | | Protein 74 gm., fat 81 gm., carbohyrate 216 gm. | | |
| 2 | 405 | 157 | 248 | −4.3 | +1.7 | 75 | Commercial. | " | " | " |
| 2 | 458 | 222 | 236 | +35.4 | −1.7 | 150 | " | " | " | " |
| 2 | 521 | 260 | 261 | +58.5 | +8.8 | 225 | " | " | " | " |
| 1 | 550 | 291 | 259 | +77.5 | +7.9 | 300 | " | " | " | " |
| 7 | 462 | 172 | 290 | | | 0 | | " | " | " |

Subject L.

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 566 | 213 | 353 | | | 75 | Commercial. | Protein 93 gm., fat 136 gm., carbohydrate 80 gm. | | |
| 1 | 608 | 240 | 368 | | | 150 | " | " | " | " |
| 1 | 724 | 304 | 420 | | | 225 | " | " | " | " |

*The Effects of Glucose Ingestion upon the Excretion of Normal Urine Sugar.*

Table I shows the amounts of sugar excreted upon the basal diet alone and when commercial and pure glucose were superimposed upon this diet. The purer glucose used was "Exose," Corn Products Refining Company. In the case of both subjects, the average values for the basal diet preceding the glucose tests, as well as the average values for the total number of days the subjects ate this diet, are given. The change in the fermentable and non-fermentable fractions is computed from the values for the basal diet immediately preceding the glucose tests.

*Subject B.*—The ingestion of pure glucose caused an increased excretion of fermentable sugar only when 300 gm. were taken. Commercial glucose gave an increase in this fraction at a lower level than did the purer substance. The excretion of non-fermentable sugar was not significantly affected by either type of glucose.

*Subject H.*—Pure and commercial glucose caused the fermentable fraction to increase. The amount of non-fermentable sugar was not significantly changed.

The results of these experiments indicate that large amounts of glucose may be superimposed upon an adequate caloric diet without greatly influencing the total sugar excretion. There was an increased excretion of fermentable sugar without significant change in the output of non-fermentable reducing substances. Commercial glucose appears to be more effective in producing an increased excretion of fermentable sugar than does the purer substance. The slight response of these two subjects to glucose ingestion emphasizes the ability of healthy individuals to metabolize carbohydrates.

*Subject L.*—This subject showed an increased output of both forms of sugar when commercial glucose was added to a diet consisting of protein 93 gm., fat 136 gm., and carbohydrate 80 gm.

These experiments taken as a whole indicate that it is possible to increase the excretion of fermentable sugar by the ingestion of glucose. This increase is not large. However, there is no question about the disturbed relationship between the fermentable

and non-fermentable fractions. Our results differ from those of Benedict, Osterberg, and Neuwirth (8) in that a consistent decrease in the absolute amount of the non-fermentable sugar was not observed.

Attention should be directed to the fact that there is a considerable daily variation in the excretion of sugar even though the diet remains constant. For the sake of completeness the highest, lowest, and average values for the basal diet are given. The findings for Subject B for 10 days were: total, 587 to 392, average, 489 mg.; fermentable, 251 to 118, average, 189 mg.; and non-fermentable, 366 to 223, average, 300 mg. The values for

TABLE II.

*Sugar Excretion, Mg. per Hour, Basal Diet.*

| Sugar. | 8–10 a.m. | 10–12 a.m. | 12–2 p.m. | 2–4 p.m. | 4–6 p.m. | 6–8 p.m. | 8–10 p.m. | 10 p.m.–8 a.m. |
|---|---|---|---|---|---|---|---|---|
| Subject B 10 days. | | | | | | | | |
| Total | 21.5 | 21.5 | 26.8 | 26.1 | 21.1 | 34.9 | 22.9 | 14.3 |
| Fermentable | 9.5 | 7.7 | 10.4 | 12.1 | 8.0 | 10.9 | 9.2 | 5.7 |
| Non-fermentable | 12.0 | 13.8 | 16.4 | 14.0 | 13.1 | 24.0 | 13.7 | 8.6 |
| Subject H 7 days. | | | | | | | | |
| Total | 19.4 | 20.2 | 21.4 | 24.2 | 20.6 | 26.1 | 23.8 | 15.1 |
| Fermentable | 6.2 | 6.5 | 8.8 | 9.6 | 8.5 | 9.5 | 10.0 | 5.4 |
| Non-fermentable | 13.2 | 13.7 | 12.6 | 14.6 | 12.1 | 16.6 | 13.8 | 9.7 |

Meals at 8 a.m., 12 m., and 6 p.m.

Subject H for 7 days were: total, 531 to 404, average, 462 mg.; fermentable, 208 to 161, average, 172 mg.; and non-fermentable, 362 to 237, average, 290 mg.

Table II gives the average excretion of sugar in mg. per hour when the two subjects were eating the basal diet. It is seen that the rate during the day was always in excess of the night rate. The phenomenon of glycuresis is shown clearly in the case of Subject B but is less prominent with the other subject. The hourly excretion rate for several of the other diets was also determined but since nothing of particular interest was noted they have not been recorded.

*The Excretion of Sugar upon Different Diets.*

In Table III are given the values for urine sugar upon various diets. The figures are arranged beginning with the lowest and ending with the highest intake of protein. It is seen that the lowest percentage of non-fermentable sugar was excreted on the smallest intake of protein and the highest percentage was excreted on the largest intake of protein. In the case of Subject

TABLE III.

*Excretion of Sugar on Various Diets.*

| Experimental days. | Sugar. | | | | | Nitrogen. | Diet. | | |
|---|---|---|---|---|---|---|---|---|---|
| | Total. | Fermentable. | Non-fermentable. | Fermentable. | Non-fermentable. | | Protein. | Carbohydrate. | Fat. |
| | | | | Subject B. | | | | | |
| | *mg.* | *mg.* | *mg.* | *per cent* | *per cent* | *gm.* | *gm.* | *gm.* | *gm.* |
| 4 | 464 | 243 | 221 | 52.4 | 47.6 | 5.89 | 33 | 235 | 133 |
| 10 | 489 | 189 | 300 | 38.7 | 61.3 | | 74 | 216 | 81 |
| 5 | 603 | 201 | 402 | 33.3 | 66.7 | 10.95 | 85 | 67 | 175 |
| 4 | 505 | 181 | 324 | 35.8 | 64.2 | 11.57 | 87 | 143 | 141 |
| 8 | 604 | 193 | 411 | 31.9 | 68.1 | 18.09 | 145 | 168 | 151 |
| | | | | Subject H. | | | | | |
| 4 | 603 | 262 | 341 | 43.4 | 56.6 | 5.15 | 33 | 235 | 133 |
| 7 | 462 | 172 | 290 | 37.2 | 62.8 | | 74 | 216 | 81 |
| 6 | 622 | 220 | 402 | 35.4 | 64.6 | 10.03 | 85 | 67 | 175 |
| 4 | 551 | 170 | 381 | 30.9 | 69.1 | 11.50 | 87 | 143 | 141 |
| 8 | 631 | 168 | 463 | 26.6 | 73.4 | 17.07 | 145 | 168 | 151 |
| | | | | Subject L. | | | | | |
| 1 | 874 | 351 | 523 | 33.1 | 66.9 | | 100 | 300 | 100 |
| 1 | 1062 | 550 | 512 | 51.8 | 48.2 | | 92 | 500 | 93 |

H there is perfect agreement inasmuch as when the protein of the diet was increased there was an increasing proportion of the sugar present in the non-fermentable form. The values for Subject B are out of line in one instance. If the figures are arranged according to carbohydrate or fat ingested, there is no relationship to be found. The absolute amounts of sugar, as well as of the

two fractions, showed variations on the different diets. These variations cannot be explained with the limited data at hand. Both subjects excreted the largest amount of fermentable sugar on the diet containing the least protein and the highest carbohydrate.

## DISCUSSION AND SUMMARY.

Our experiments show that the addition of glucose to a standard diet causes an increased excretion of fermentable sugar without any significant change in the non-fermentable fraction. Commercial glucose appears to be somewhat more effective than the purer substance in producing this change. The increase in the excretion of fermentable sugar was not large. These results illustrate the great capacity of strictly normal individuals for metabolizing carbohydrate materials.

Study of the sugar excretion on different diets revealed what appears to be a significant relationship between the protein of the food and the non-fermentable sugar of the urine. The values showed an increasing percentage of non-fermentable sugar as the protein intake was augmented. Attention has been called to the daily variations in sugar excretion even when the diet is constant. Therefore, we are not inclined to state without qualification that the non-fermentable sugar is derived from protein metabolism. More extensive data than ours will be necessary to prove this point. We believe, however, that our experiments indicate that such is the chief source of this fraction of the normal urine sugar.

In conclusion, it may be stated that our results indicate the chief source of the fermentable sugar to be the main carbohydrate metabolism. The non-fermentable reducing substances appear to be largely dependent upon the protein metabolism.

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# THE USE OF THE QUINHYDRONE ELECTRODE FOR THE DETERMINATION OF THE HYDRION CONCENTRATION OF FECES.*

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(Received for publication, September 24, 1925.)

Since its description by Biilmann (1) the quinhydrone electrode has been found suitable for the determination of the reaction of many kinds of solutions varying from soil extracts (2) to blood plasma (3). We have used it for the determination of fecal reactions and have found it quite as reliable and much more convenient than the hydrogen electrode with which it has been compared. The results reported below represent the agreement usually obtained between duplicates with the same electrode and with the two different electrodes.

The determinations with the hydrogen electrode were carried out as described in a former paper (4) except that the system was standardized against 0.1 N HCl as recommended by Cullen (5). His recommendation in this regard, made in connection with his study of blood plasma (3), was also followed with the quinhydrone electrode. Gold plated platinum electrodes, platinized in the case of the hydrogen electrodes, were used. The same cells were used for both methods. The feces were macerated to a thin, homogeneous paste with water and poured into the cell. In the case of the hydrogen electrode, hydrogen was passed into it in the usual way. In the case of the quinhydrone electrode, a little quinhydrone was added, the cell and contents shaken until the quinhydrone was uniformally distributed throughout the liquid,

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and the reading taken at once. The temperature was read and the pH calculated by means of Cullen's equation,

$$\text{pH} = \frac{\text{E.M.F.} + e_q}{0.0001984\,T}$$

$e_q$ being the E.M.F. of the quinhydrone electrode found by standardizing the system against a solution of known pH.

The results are given in Table I.

TABLE I.

*Fecal Reactions.*

| Sample No. | Temperature. | pH. Hydrogen electrode. | | | pH. Quinhydrone electrode. | | | Averages $pH_H - pH_Q$. |
|---|---|---|---|---|---|---|---|---|
| | | a | b | (a − b) | a | b | (a − b) | |
| | °C. | | | | | | | |
| 7252 | 25 | 6.86 | | | 7.02 | 7.01 | 0.01 | 0.16 |
| 7271 | 24 | 4.98 | 4.91 | 0.07 | 5.20 | 5.22 | 0.02 | 0.27 |
| 7272 | 24 | 6.56 | 6.46 | 0.10 | 6.64 | 6.64 | 0.00 | 0.13 |
| 7281 | 20 | 6.79 | 6.79 | 0.00 | 6.88 | 6.88 | 0.00 | 0.09 |
| 7282 | 20 | 6.63 | 6.53 | 0.10 | 6.60 | 6.60 | 0.00 | 0.02 |
| 7291 | 23 | 7.18 | 7.06 | 0.12 | 7.25 | 7.23 | 0.02 | 0.12 |
| 7292 | 23 | 7.25 | 7.15 | 0.10 | 7.30 | 7.30 | 0.00 | 0.10 |
| 7301 | 23 | 7.35 | 7.35 | 0.00 | 7.23 | 7.32 | 0.09 | 0.07 |
| 7302 | 23 | 6.84 | 6.84 | 0.00 | 6.84 | 6.84 | 0.00 | 0.00 |
| 7311 | 23 | 7.49 | 7.57 | 0.08 | 7.52 | 7.56 | 0.04 | 0.01 |
| 811 | 22 | 7.62 | 7.56 | 0.06 | 7.69 | 7.69 | 0.00 | 0.10 |
| 812 | 22 | 7.17 | 7.17 | 0.00 | 7.20 | 7.22 | 0.02 | 0.04 |
| 831 | 24 | 7.42 | 7.40 | 0.02 | 7.41 | 7.42 | 0.01 | 0.01 |
| 832 | 24 | 7.00 | 7.08 | 0.08 | 7.03 | 7.10 | 0.07 | 0.02 |
| 841 | 25 | 7.50 | 7.50 | 0.00 | 7.37 | 7.35 | 0.02 | 0.14 |
| 842 | 25 | 7.10 | 7.07 | 0.03 | 7.08 | 7.07 | 0.01 | 0.01 |
| 851 | 25 | 7.77 | 7.77 | 0.00 | 7.60 | 7.63 | 0.03 | 0.15 |
| 852 | 25 | 6.73 | 6.70 | 0.03 | 6.67 | 6.67 | 0.00 | 0.05 |
| 871 | 26 | 6.88 | | | 6.83 | 6.83 | 0.00 | 0.05 |
| 872 | 26 | 6.82 | 6.79 | 0.03 | 6.78 | 6.78 | 0.00 | 0.03 |
| 891 | 26 | 7.02 | 7.00 | 0.02 | 6.89 | 6.94 | 0.05 | 0.08 |
| 892 | 26 | 6.38 | 6.23 | 0.15 | 6.42 | 6.42 | 0.00 | 0.11 |

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# THE NUTRITIONAL REQUIREMENT OF THE CHICKEN.

## VI. DOES THE CHICKEN REQUIRE VITAMIN C?*

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(Received for publication, October 13, 1925.)

In 1922 (1) we stated that the water-soluble and antiscorbutic vitamin requirement of chicks can be met by the amounts of these substances contained in a cereal grain and skimmed milk. Further experiments in chick feeding have fully confirmed this statement. On rations made from the grains supplemented with raw liquid skimmed milk or with milk powder no evidence of scurvy in the chicken develops. However, from such experiments it is always possible to conclude that sufficient antiscorbutic vitamin was contained in the ration to protect the chick, although such a ration would not protect the guinea pig from scurvy, unless the milk consumption was liberal.

In order to study the problem more fully, it was decided to rear chicks on rations either freed from the antiscorbutic vitamin by heat or on rations made of purified food materials and presumably free from vitamin C. Should there be an accumulation of the antiscorbutic vitamin in the liver of chicks so reared, then such evidence would strongly support the idea that the chick does not require vitamin C preformed in the diet in the same sense as does the guinea pig, but in all probability can build it from certain dietary complexes.

The method used in testing the accumulation of vitamin C in

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chicken livers was to feed these livers at different levels of intake to scorbutic guinea pigs. This method had already been used by Parsons (2), Parsons and Hutton (3), and Lepkovsky and Nelson (4) in their study of the accumulation of vitamin C in rat livers. Recently Carrick and Hauge (5) have used this method in studies of the accumulation of vitamin C in the livers of chicks fed scorbutic diets and reached conclusions similar to those announced by us earlier (1) and also in agreement with the facts presented in this paper.

## EXPERIMENTAL.

The accumulation of vitamin C in the livers of animals on scorbutic rations may be explained in several ways; it may represent a power on the part of the animal to concentrate vitamin C in its liver when present in minute amounts in the ration; it may represent a power on the part of the animal to activate some inactive precursor; or it may represent a power on the part of the animal to synthesize this vitamin. The experimental animals whose livers are to be tested should be on the scorbutic ration the shortest time possible so as to provide the least opportunity for concentration in their livers of any of this vitamin that may be contained in the ration. Carrick and Hauge (5) are the only workers who have published data on the potency of chicken livers in respect to vitamin C when fed on a scorbutic diet. They used a group of cockerels of the American variety which had been fed on a ration consisting of degerminated yellow corn, tankage, yeast, and a salt mixture. The birds fed on this ration were used in other experiments on polyneuritis, but were completely cured before being used for the scurvy test. The length of time the chickens were on the experimental ration is not stated, but this is a very important factor in such studies. We cannot by any means say that yellow corn, tankage, and yeast are absolutely free from vitamin C. That guinea pigs succumb to scurvy on such a ration simply shows that there is insufficient vitamin C present in the ration to protect guinea pigs from scurvy; it does not disprove the presence in the ration of minute amounts of vitamin C which might very well be concentrated in chicken livers over a long period of feeding.

On November 29 a group of Plymouth Rocks was started on the "Wisconsin Chick Ration" (6) consisting of 80 parts of ground

yellow corn, 20 parts of standard wheat middlings, 5 parts of raw bone, 5 parts of pearl grit (crushed marble), 1 part of common salt, and skimmed milk *ad libitum*. The skimmed milk was autoclaved for 1 hour at 15 pounds pressure in order to destroy vitamin C. In addition the birds were irradiated 10 minutes daily with ultra-violet light to prevent leg weakness or rickets.

On this ration, which can be considered a fairly good scorbutic ration, the chickens grew very well and on February 11, the feeding of livers from these chickens began. The chickens were on the Wisconsin Chick Ration for 73 days when the liver feeding began; they were killed daily; their livers were removed and passed through a wire screen to remove the connective tissue and vascular materials and the remaining liver pulp fed to scorbutic guinea pigs from a syringe at levels of 1 to 3 gm. daily. The guinea pigs were brought down with scurvy on our scurvy-producing ration consisting of 69 parts of rolled oats (coarsely ground), 25 parts of alfalfa meal heated for 30 minutes at 15 pounds steam pressure, 5 parts of crude casein, and 1 part of common salt.

The guinea pigs receiving 1 gm. of liver daily showed some improvement but were not completely cured nor did they gain weight (see Chart I); after 17 days of liver feeding they still showed signs of scurvy, indicating that 1 gm. of this chicken liver fed daily was just on the scorbutic border-line and somewhat insufficient for a complete cure. The guinea pigs receiving 3 gm. of liver were cured at once, as can be seen in Chart I.

The results secured with the above ration of natural materials, particularly the grain portion, is open to the same criticism which we made of the results obtained by Carrick and Hauge (5); namely, that the ration may have contained traces of vitamin C which could be concentrated in the liver of the chickens. It has been pointed out by Eddy, Kohman, and Carlsson (7) and by Cavanaugh, Dutcher, and Hall (8) that natural food materials may be subjected under certain conditions to high temperatures for considerable lengths of time without destroying vitamin C. Consequently, we resorted to the use of a ration composed of purified food materials and which would be as free from vitamin C as can be prepared in any considerable quantity. Further, it is entirely possible to grow chickens on purified diets in which the presence of vitamin C must be greatly reduced, if present at all.

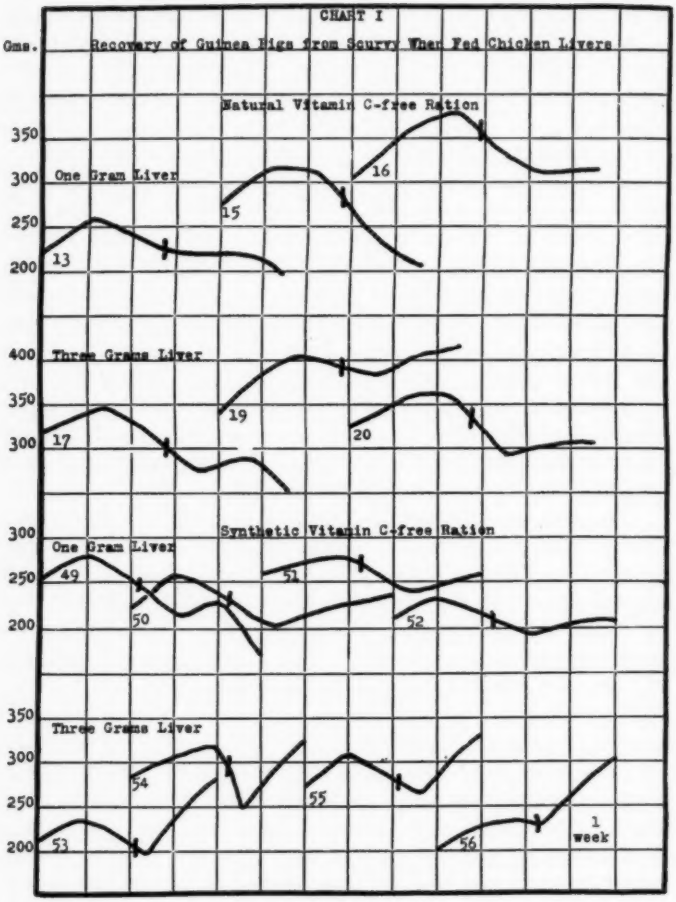


CHART 1.

On March 10 a group of Rhode Island Reds was started on the following synthetic ration:

| | *per cent* |
|---|---|
| Dextrin | 65 |
| Casein | 18 |

| | |
|---|---|
| Yeast | 10 |
| Salt Mixture 32 | 5 |
| Agar | 2 |
| Saponified cod liver oil (equivalent to 5 per cent of raw oil). | |

The dextrin was prepared by moistening corn-starch with water and heating it for 1 hour at 15 pounds pressure. It was then dried at 60–70°C. for at least 2 weeks and ground. The agar was dried at 60–70°C. for 2 months and ground. The casein was extracted with 95 per cent alcohol for 1 week by letting it soak in the alcohol in large percolators. The alcohol was drawn from the casein every day and a fresh supply immediately added. This was continued for 1 week. The casein was then dried at 60–70°C. for 24 hours. The yeast was dried brewers' yeast. The temperature to which it had been exposed was unknown to us. The cod liver oil was saponified by boiling 275 cc. of the oil in 500 cc. of 20 per cent KOH, cooling, and diluting with 2000 to 3000 cc. of water. The mass was then extracted with ether, two extractions of about 500 cc. each being made. Portions of the ether extract were evaporated on the ration in order to make the amount introduced equivalent to approximately 5 per cent as raw oil. Salt Mixture 32 (9) was used in the make-up of the ration.

It was felt that this ration was as free from vitamin C as can at present be conveniently prepared in any quantity and yet obtain fairly good growth of chicks. Every constituent of the ration with the exception of the salts had been subjected to considerable heat in the presence of oxygen, which is an effective combination for the destruction or inactivation of vitamin C. The chickens grew rather slowly on this ration and were somewhat irregular in size and weight. On June 2 (84 days after the chicks were started) they were killed daily as needed and their livers fed to scorbutic guinea pigs. The chickens weighed about 300 to 600 gm. with an average of about 450 gm. when killed. As can be seen from Chart I, 3 gm. of the liver fed daily cured scorbutic guinea pigs at once and 1 gm. of the liver fed daily improved scorbutic guinea pigs sufficiently so that they even gained slightly in weight; but when discontinued they still showed unmistakable signs of scurvy. It is fairly certain then that livers from chickens fed on a purified diet as free from vitamin C as it was possible to prepare readily in considerable amounts and suitable for the

growth of chickens contained as much vitamin C as livers from chickens fed on a diet of natural foodstuffs. Apparently the chicken, like the rat, does not need vitamin C preformed in the diet in the same sense as does a guinea pig; but its presence in large amounts in the livers of chickens even on synthetic diets indicates that it plays some rôle in its metabolism. Whether the chicken synthesizes this vitamin from an inactive precursor or from some other organic complex cannot at present be stated.

SUMMARY.

1. Chicks fed a ration of grains and heated skimmed milk, presumably free or at least very low in its content of vitamin C, did not suffer from scurvy. Further, their livers were an abundant source of vitamin C which, when fed at a level of 3 gm. daily, cured guinea pigs suffering from scurvy.

2. Chicks fed a ration of purified food materials—dextrin, casein, salts, etc.—did not develop scurvy. The livers from these chickens were also potent sources of vitamin C.

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# ULTRA-VIOLET ABSORPTION SPECTRA OF BLOOD SERUM AND CERTAIN AMINO ACIDS.

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*(From the State Institute for the Study of Malignant Disease, Buffalo.)*

(Received for publication, September 26, 1925.)

Blood serum produces a characteristic absorption curve in the ultra-violet part of the spectrum. It has been proposed in several papers (1) that changes in this curve may give us valuable information about pathological conditions. Before we try to establish such relations between the change of the curve and the pathology we ought to know what it is that produces the absorption and which chemical and physical factors will influence it. Lewis (2) has shown that the absorption is due mainly to the proteins in the serum. In a recent article (1) we have stated that it is the tryptophane and tyrosine constituents of the protein molecules which are responsible for the characteristic band around 2800 Å. We will here describe how we arrived at this conclusion and relate some investigations concerning the absorption process.

For this study we used a Hilger quartz spectrograph size C with a Hilger sector photometer. As light source we used an under-water spark between tungsten electrodes arranged after the description in Scientific Papers of Bureau of Standards, No. 440 (the method was originated by Howe (3)).

In order to make our absorption curves comparable to those of Lewis we used for blood serum a chamber 0.1 mm. deep filled with undiluted serum or a ½ cm. chamber filled with 1 part serum and 49 parts of water. Fig. 1 shows two characteristic curves for human serum when the wave-length $\lambda$ in Ångström units is used as abscissa and the molecular extinction coefficient ($E$) times the number of mols ($C$) divided by the number of liters of solution as ordinate. It has long been known that cyclic compounds as a rule give absorption bands in the ultra-violet region between 2200 and 2900 Å. Each one of these compounds gives a characteristic

absorption curve which can be used to identify it. The curve is, however, dependent upon the solvent. All the compounds used for this investigation were dissolved in water. Few aliphatic compounds give an absorption band in the region mentioned. (Many of them give, however, a continuous absorption below 2100 Å.). Neither do all the proteins give an absorption band around 2800 Å. For gelatin, for instance, we found the absorption curve reproduced in Fig. 2. It was illuminating to find that tyrosine and tryptophane give absorption bands around 2800 Å. (4).

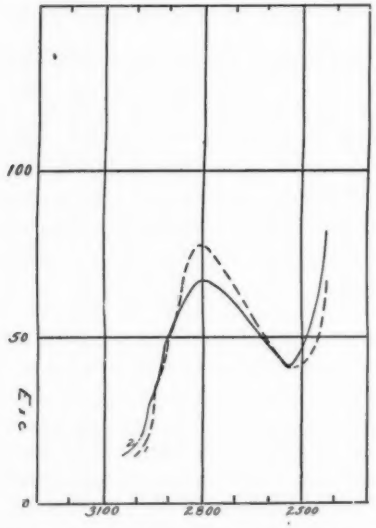



FIG. 1. Curves 1 and 2, human blood serum.

These amino acids are constituents of serum albumin and serum globulin but not of gelatin. It was, therefore, reasonable to assume that these amino acids were at least partly responsible for the absorption band of the blood serum. We then wanted to find the quantitative relation between the absorption of the individual amino acids and the blood serum. For this purpose we could not use the absorption curves of Ward and others but had to make new determinations.

From the analysis of Abderhalden and Kiyotaki we estimated

that blood serum contains approximately 0.415 per cent tyrosine and 0.169 per cent tryptophane as constituents of the proteins. Using corresponding dilutions of the amino acids in the same absorption chamber as had been used for blood serum (0.1 mm. depth) or 50 times this dilution in a 50 times deeper chamber, the curve in Fig. 3 was obtained for tryptophane and Curve 1 in Fig. 4 for tyrosine. These curves indicate that the characteristic band of blood serum around 2800 Å. is produced mainly by

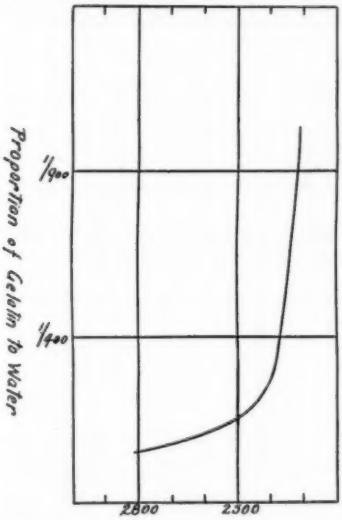



FIG. 2. Gelatin in water. 1 cm. cell. Intensity reduced to $\frac{1}{10}$.

tyrosine and tryptophane and that the other amino acids modify the band slightly and add to the absorption below 2500 Å. A mixture of tyrosine, tryptophane, phenylalanine, cystine, glycine, leucine, and glutamic acid in indicated proportions gives Curve 1 in Fig. 5. This curve agrees fairly well both qualitatively and quantitatively with Curve 1, Fig. 6, representing the absorption of blood serum. It is, however, shifted over slightly towards shorter wave-lengths and shows more absorption, as can be seen in Fig. 7.

There might be several reasons for this shift and we considered

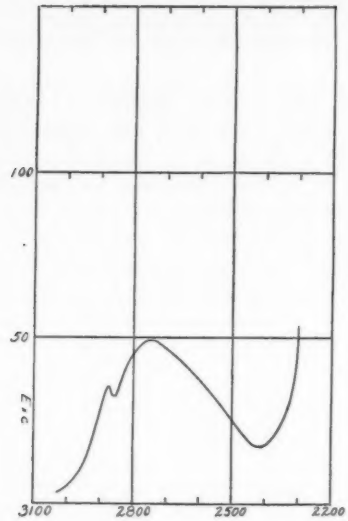



FIG. 3. Tryptophane. pH 4.0 and 12.7. 0.034 gm. in 1000 cc. of water ½ cm. cell (equivalent to 0.17 gm. per 100 cc. with a 0.01 cm. cell).

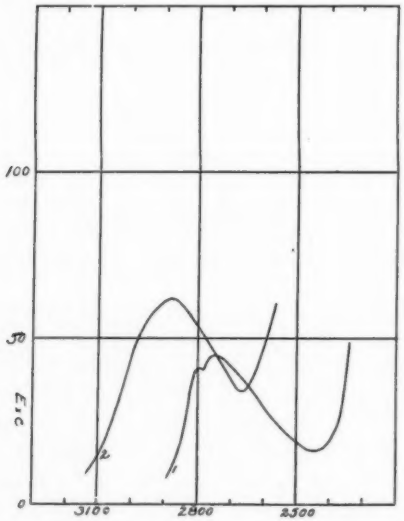



FIG. 4. Curve 1, tyrosine pH 4.0. Curve 2, tyrosine pH 12.7. 0.0041 gm. in 100 cc. of water 1 cm. cell (equivalent to 0.41 gm. in 100 cc. with a 0.01 cm. cell).

the following three. (1) Tadokoro (5) had found that certain salt solutions influence the absorption of blood sera. (2) It seemed probable that the hydrogen ion concentration should have some influence on the absorption as we know that it is responsible for the change of color of indicators. (3) Numerous investigations have shown the following relations between organic compounds and

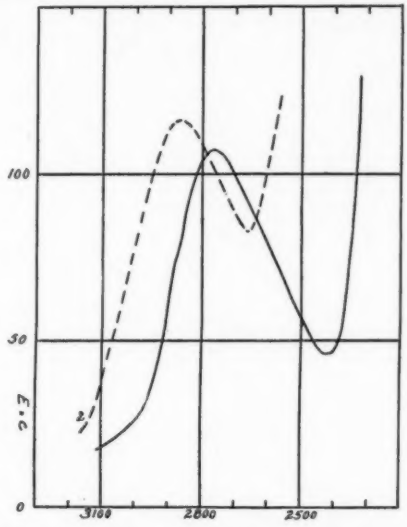



FIG. 5. Curve 1, solution of amino acids. pH 7.5. Curve 2, solution of amino acids. pH 12.7.

| | gm. |
|---|---|
| Phenylalanine | 0.023 |
| Tyrosine | 0.0415 |
| Cystine | 0.0120 |
| Tryptophane | 0.0169 |
| Glycine | 0.0099 |
| Leucine | 0.1331 |
| Glutamic acid | 0.05493 |

in 1000 cc. 1 cm. cell (equivalent to ten times these amounts of amino acids in 100 cc. water when used in a 0.01 cm. cell).

absorption. If a certain cyclic compound with a side chain gives a single absorption band in the ultra-violet then a similar compound with a longer side chain for instance, with an additional

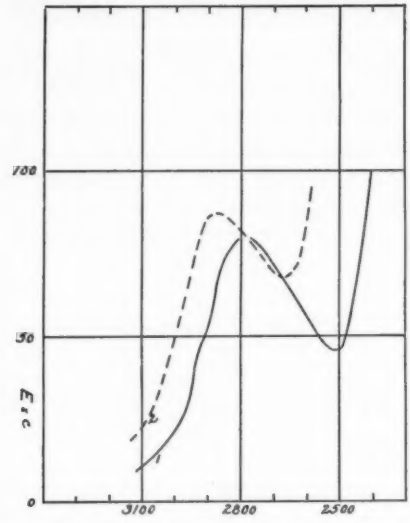



FIG. 6. Curve 1, human blood serum. pH 7.5. Curve 2, human blood serum + NaOH. pH 12.7. Used undiluted with a 0.01 cm. cell.

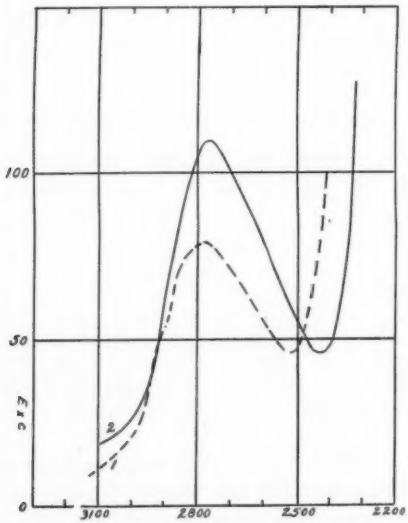



FIG. 7. Curve 1, human blood serum. pH 7.5. Curve 2, solution of amino acids. pH 7.5.

$CH_2$ group, gives a similar absorption band which is shifted slightly towards longer wave-lengths. It seemed possible that the combination of amino acids in a protein might produce a similar shift in the band produced by the amino acids.

1. We found that blood sera gave the same absorption band whether they were diluted with saline solution or with pure water. Blood albumin also gave the same curve whether dissolved in water or saline solution. The amino acids gave the same absorp-

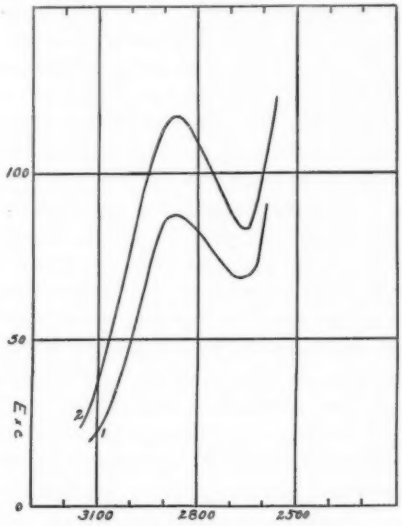
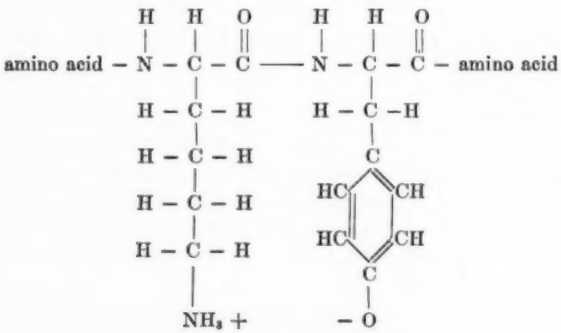



Fig. 8. Curve 1, human blood serum. pH 12.7. Curve 2, solution of amino acids. pH 12.7.

tion curve with and without the NaCl. In order to produce a measurable change of the absorption curve for blood serum, it was necessary to add an appreciable amount of $ZnSO_4$ and even then no shift was noticed, just an increase in the absorption. It seems, therefore, improbable that the difference between the amino acid curve and the blood serum curve could be referred to the difference in the amount of salt present.

2. Both the curves for the blood serum and for the mixture of amino acids referred to above were taken at a pH 7.5 (Fig. 7).

When the pH was changed for the mixture to 12.7 then the curve shifted to Curve 2 in Fig. 5. This shift was in the right direction but it was too great to make the curve agree with the blood serum curve. However, when the blood serum was made alkaline to pH 12.7, then its curve shifted to Curve 2 in Fig. 6 and for this pH the mixture curve agreed qualitatively with it as shown in Fig. 8. If the concentrations of the amino acids were reduced 25 per cent then the two curves would agree also quantitatively within the limits of experimental errors. When the amino acids were studied separately it was found that the tryptophane did not show a shift when it was made alkaline, while tyrosine showed a shift from Curve 1 to Curve 2 in Fig. 4 when the pH was increased from 4.0 to 12.7. The reason for this we believe is that an OH group is connected to the benzene ring in tyrosine but not in tryptophane. In another article (6) we will give the reason for this assumption and also show why it may be assumed that Curve 2 in Fig. 4 is produced by the tyrosine ionized at this hydroxyl group, while Curve 1 is produced by other forms of tyrosine. The difference between the "mixture curve" and the blood serum curve at pH 7.5 seems to indicate that the tyrosine constituent of the blood serum is partly ionized at this hydroxyl group in a neutral or even weak acid solution. If the amino acids are chained together in the proteins in the ordinarily assumed way, we may perhaps imagine that an amino group from one constituent is placed near the hydroxyl group of tyrosine and ionize it by robbing it of its H. For instance




Lysine. Tyrosine.

3. The last assumption stated in (2) is the only one we need to make concerning the influence on the absorption of the combination of amino acids to proteins. However, it would be of importance if the influence of the combination were tested directly. We tried to do this by hydrolyzing the proteins of blood serum. Evidently the tyrosine and tryptophane were broken up during this procedure, as the absorption around 2800 Å. of the hydrolyzed serum did not show up clearly.

SUMMARY.

1. The absorption band around 2800 Å. of blood serum is due to the proteins present and it is mainly the tyrosine and tryptophane constituents of the proteins which are responsible for this band.

2. The absorption band of tyrosine shifts towards longer wavelengths and its intensity is increased when the solution is made alkaline, as shown in Fig. 4. The absorption of blood serum changes in a similar manner but to a smaller extent when the hydrogen ion concentration is reduced below $10^{-12.7}$, as is shown in Fig. 6.

3. A mixture of tyrosine, tryptophane, phenylalanine, cystine, glycine, leucine, and glutamic acid in the proportion indicated by analysis of blood serum (albumin and globulin) gives the absorption curves shown in Fig. 5. Curve 2 at a pH 12.7 agrees qualitatively with the blood serum curve taken at the same hydrogen ion concentration. A quantitative agreement between the two curves within the experimental errors could be arrived at if the concentrations of the mixed amino acids were reduced 25 per cent.

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# THE EFFECT OF ORANGE JUICE ON THE CALCIUM, PHOSPHORUS, MAGNESIUM, AND NITROGEN RETENTION AND URINARY ORGANIC ACIDS OF GROWING CHILDREN.*

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The purpose of this investigation was to observe the effect on mineral and nitrogen metabolism of the addition of orange juice to the diet of growing children. Observations were also made on the urinary organic acids, ammonia, and hydrogen ion concentration. As oranges have been shown to produce an improved condition in the underweight child and as the retention of minerals and nitrogen by the growing organism is considered an indication of normality and health, it has seemed possible that oranges might function by favoring a better assimilation.

## REVIEW OF LITERATURE.

*Orange Juice and Growth in Children.*—That oranges are of value in causing a gain in weight of malnourished children has been demonstrated by Newell and Miller (1) who worked with fourteen underweight children chosen because they had shown little or no improvement as a result of group instruction in health habits. When 45 cc. of orange juice were added daily to the diet of these children and all other conditions were kept unchanged, the percentage of expected gain of the group was increased from 49 for the 4 months preceding the experiment to 132 during the 3 months in which orange juice was fed. Another study (2) made in California on 256 malnourished children demonstrated a marked stimulation to growth when oranges were fed as a mid-morning lunch. During 2 months in the winter those children who received an orange a day, in

* The data are taken from the thesis of Margaret S. Chaney submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, in the Ogden Graduate School of Science, University of Chicago, 1925.

addition to their normal diet, gained an average of 141 per cent above the predicted, while the children who received no mid-morning lunch gained 28 per cent above the expected amount. In the spring the comparative gain above that predicted was 118 for the orange group as against 18 for the controls.

*Components of Orange Juice Which Have Been Shown to Affect Metabolism.*—The orange has several components which are of value in regulating metabolism. All of the commonly accepted vitamins have been shown to be present in oranges. Givens and McClugage (3) state that oranges head the list of foods protecting against scurvy, and Sherman (4) goes so far as to say that "better growth, higher stamina, and better general health and disposition are induced by the feeding of vitamin C in the form of orange or tomato juice." Investigations also show the potency of oranges in the fat-soluble vitamin (5, 6, 7).

The importance of the antineuritic factor in oranges was demonstrated by Byfield and Daniels (8) who observed that babies not gaining normally on the prescribed diet of modified milk and 15 cc. of orange juice daily, received a marked stimulation to growth when the juice was increased to 45 cc., and they proved that the acceleration in growth was due to a relatively large amount of water-soluble B in the fruit, as a similar growth was not observed when this vitamin was removed. Hess (9) likewise got a gain in weight in infants when oranges were added to the diet; the omission of orange juice was accompanied by a period of stationary weight.

The organic constituents of oranges may aid by supplementing a deficiency of minerals in other foods; or by causing a shift in acid-base balance from the acid condition of a diet high in meat and cereal foods. Oranges yield a residue basic in character, 5.61 cc. normal base in 100 gm. of orange juice as calculated by Sherman and Gettler (10). Blatherwick and Long (11) found that the ingestion of from 600 to 2400 cc. of orange juice daily increased the pH and the organic acids of the urine and diminished the ammonia.

## *Some Factors Influencing the Retention of Minerals by the Body.*

*Vitamins.*—Investigations on the relationship between the intake of vitamins A, B, and C and calcium retention have lead to no marked or consistent conclusions, although a slight favorable influence on calcium metabolism was observed by Miyadera (12) in growing dogs when butter and germinating grains were used instead of the lard and wheat of the basal diet, and by Bogert and Trail (13) in adult women when butter fat or yeast was added to a practically vitamin-free diet. Recently the special antirachitic factor or vitamin which decidedly influences calcium deposition has been demonstrated (14), and many detailed studies made. Orange juice has not been proved to contain it.

*Acidity of the Gastrointestinal Contents.*—The hydrogen ion concentration of the contents of the stomach and upper small intestine controls to a large extent the absorption of calcium and phosphorus. Zucker, Johnson,

and Barnett (15) have found that a diet favorable to the absorption of these elements could be changed to one producing rickets by the addition of alkali. According to Telfer (16) absorption of calcium is hindered by achlorhydria, by hypoacidity, or by a pathological state in the upper small intestine. Jones (17) has successfully used hydrochloric acid therapy in rickets.

*Acid-Base Ratio.*—A diet giving an alkaline residue in metabolism, according to Shohl (18) is essential in infancy where the body is rapidly developing. Forbes and Keith (19), in a complete review of the work done on phosphorus metabolism up to 1914, conclude that acid formation in the body increases calcium and phosphorus excretion especially in the urine, and that the ingestion of acids and acid salts results in a diminished base balance. Experiments by Bogert and Kirkpatrick (20) on adult women showed a somewhat better calcium retention on a base-forming diet than on a balanced or acid-forming one. Steenbock, Nelson, and Hart (21) have found decalcification of the bones in calves and swine on a high ingestion of acid-forming foods but noted no serious effect on growth and reproduction.

Several complete acid-base metabolism studies have been made to determine the effect of acid and alkali on mineral retention by the body. Baumann and Howard (22) on adding orange juice to the diet of a man sick with scurvy found a greater retention of calcium, magnesium, sodium, potassium, chlorine, and nitrogen, but the phosphorus stayed in negative balance throughout. In order to determine the effect of acidosis on the retention of minerals, Sawyer, Baumann, and Stevens (23) substituted fat for an equal number of calories of carbohydrate in the diet of two normal children, boys 5 and 8 years old, with the result that the alkali and nitrogen retention was much reduced.

In a complete acid-base metabolism experiment on normal infants Shohl and Sato (24) observed that the normal positive base balance is 10.0 cc. $\pm$ 2 cc. of 0.1 N per kilo per day; acid added to the diet of these infants caused a smaller mineral retention than normal, and alkali a salt retention more alkaline in character.

*Intake as Related to Output.*—A metabolism experiment conducted by Sherman and Hawley (25) on children 3 to 13 years of age shows that on an adequate intake of calcium an average of 0.01 gm. of calcium per kilo per day is stored regardless of the age of the child. The amount of calcium retained was shown to vary with the milk (and calcium) intake, the optimum retention amounting to 0.017 gm. per kilo per day when 1000 cc. milk were taken. The phosphorus retention was slightly less than the calcium, 0.008 gm. per kilo per day.

Little has been written concerning the magnesium requirement of man but the consensus of opinion is that if the calcium quota is sufficient the magnesium demand of the body will also be met. Bogert and McKittrick (26) concluded from metabolism experiments on four adults that the magnesium requirement of the body is probably somewhat lower than the calcium.

*Calcium-Phosphorus Ratio.*—The proportion of calcium to phosphorus in the diet is important in mineral retention. It has been demonstrated on cattle (27) and on rats (28) that an excess of either element in the diet will cause an increase in the concentration of that element in blood plasma, and a decrease in the other. In experiments on infants by Orr, Holt, Wilkins, and Boone (29) and on children by Schabad (30) similar results were obtained—an excess of sodium phosphate improving phosphorus retention and impairing calcium assimilation, while calcium chloride caused a diminution in phosphorus retention.

## EXPERIMENTAL WORK.

In order to ascertain the effect of orange juice on growing children a metabolism study was conducted without and with oranges in the diet, the retention by the body of calcium, phosphorus, magnesium, and nitrogen determined during both periods, and the urinary organic acids, ammonia, and hydrogen ion concentration observed daily.

*General Procedure.*—Two girls, 10 and 11 years old, from the Chicago Home for Jewish Orphans were selected as being normal subjects with no known organic trouble shown by a medical examination, with some indications of malnutrition, but "free to gain." Two experiments were conducted, the second a duplicate of the first, lasting respectively from April 25 to May 5, 1924, and from July 18 to August 10, 1924. During the two periods the children lived away from the Home under the constant care of one of the writers (M. S. C.) and led a regular, happy life with plenty of sunshine, outdoor air, and sleep.

Each experiment was divided into a non-orange juice and an orange juice period. The procedure was to give the children the experimental diet for a preliminary 3 days in the first experiment and 5 in the second, in order to attain equilibrium. Next, for 3 days the basal diet was continued and corresponding urine and feces collected. Then after the necessary delay of a day or two for the lag in the feces, 600 cc. to 700 cc. of the unstrained juice of California Valencia oranges were added to the diet and the experiment repeated. The diet was continued until the last collection of feces.

*Diet.*—The basal diet during the two experiments consisted of oatmeal, rice, Irish potatoes, white bread, flour, sugar, filtered oleomargarine fat, ground lean beef, and Klim whole milk powder.

C.P. sodium chloride was kept constant (1.6 gm. per child per day) because of the possibility of its affecting the calcium output (31). Distilled water was used *ad libitum* for drinking, and also for cooking purposes. Table I gives the detail of the food eaten by the children during the preliminary and orange juice periods of one of the experiments. The basal diet was less varied but similar to that to which the children were accustomed in the Home, which was largely carbohydrate, with an adequate supply of calories and protein, a low fat, mineral, and vitamin quota, and a decidedly acid residue as computed by use of Sherman and Gettler's figures (10). In the experimental diet the fat was increased over the Home diet so as to cause a good absorption of calcium, and was kept uniform in amount (32). The food of the non-orange juice period was acid-forming but the addition of oranges changed it to one predominantly basic.

*Collection of Urine and Feces.*—The 24 hour urines were collected separately, beginning with the first meal of the test, and preserved with toluene in Pyrex flasks on ice. After measuring and making up to volume, a part was removed for the nitrogen and other analyses to be done immediately, and an aliquot set aside to be combined with the other two days collections, for mineral analyses. For the marking of stools, capsules, each containing 0.2 gm. of purified wood charcoal which had been thoroughly washed with acid to remove mineral matter, were taken at the beginning and end of each collection period. The feces immediately upon obtaining were dried to constant weight on the water bath, alcohol being added during the drying, then ground, thoroughly mixed, and preserved in glass-stoppered bottles in desiccators.

*Chemical Analyses.*—Oatmeal, rice, flour, and milk powder were used for analysis as purchased without further drying. In order to obtain a representative sample of the ground beef and riced potatoes, a quantity of each sufficient for the 3 day diet was prepared, and a sample of it dried to constant weight in an electric oven at a temperature below 100°C. A slice of bread from each loaf was likewise dried. The orange juice was first evaporated to a thick syrup on a water bath and was then dried in the oven. The dried food samples were pulverized finely and preserved in air-tight containers. The oleomargarine fat and sugar were not analyzed as they were considered 100 per cent pure; and

TABLE I.

| Food. | Measure. | Weight. | Calories.* | Protein.† | Fat. | Excess in residue.‡ | |
|---|---|---|---|---|---|---|---|
| | | | | | | Acid. | Base. |
| **Experimental diet, Period 1, without oranges.** | | | | | | | |
| | | *gm.* | | *gm.* | *gm.* | *cc.N* | *cc.N* |
| Breakfast: | | | | | | | |
| Oatmeal | ¾ cup. | 20 | 80 | 3.2 | 1.4 | 2.6 | |
| Milk | ½ " | 50 | 33 | 1.7 | 2.0 | | 1.2 |
| Sugar | 2 teaspoons. | 10 | 40 | | | | |
| Bread | 3 slices. | 82 | 216 | 7.5 | 1.1 | 5.8 | |
| Oleomargarine fat | 1 tablespoon. | 12 | 100 | | 12.0 | | |
| Milk | 1 cup. | 150 | 105 | 5.0 | 6.0 | | 3.6 |
| Dinner: | | | | | | | |
| Beef | 1 cake. | 100 | 150 | 23.2 | 2.5 | 13.9 | |
| Potato | 1 cup. | 200 | 166 | 4.4 | 0.2 | | 14.4 |
| Milk* | ¼ " | 40 | 26 | 1.3 | 1.6 | | 0.9 |
| Bread | 3 slices. | 76 | 200 | 7.0 | 1.0 | 5.4 | |
| Supper: | | | | | | | |
| Rice | ½ cup. | 37 | 130 | 3.0 | 0.1 | 3.0 | |
| Bread | 5 slices. | 212 | 558 | 19.5 | 2.8 | 15.1 | |
| Flour | 1 teaspoon. | 8 | 18 | 0.9 | 1.1 | 0.8 | |
| Oleomargarine fat | 1 tablespoon. | 14 | 100 | | 14.0 | | |
| Milk | 1½ cups. | 200 | 140 | 6.6 | 8.0 | | 4.7 |
| Total | | | 2062 | 83.3 | 52.7 | 46.6 | 24.8 |
| **Experimental diet, Period 2, with oranges.** | | | | | | | |
| Foods except bread and orange juice as in Period 1 | | | 1088 | 49.2 | 47.9 | 20.3 | 24.8 |
| Bread | 11 slices. | 353 | 928 | 32.5 | 4.6 | 25.1 | |
| Orange juice | 3½ cups. | 700 | 350 | 5.6 | 1.4 | | 39.3 |
| Total | | | 2366 | 87.3 | 53.9 | 45.4 | 64.1 |

* Calculations from Bulletin 28 (37).

† Analysis by M.S.C.

‡ Calculations from Sherman and Gettler (10).

the phosphorus in flour and rice, and the calcium and magnesium in flour, rice, oatmeal, and beef were not determined because of the extremely low percentage of the element in the food and the small amounts eaten. It was computed that in the foods not analyzed a possible variation from the values given by Sherman (33) would have no appreciable effect on the results.

Calcium and magnesium were determined by McCrudden's method (34), but using methyl red as an indicator in order to ascertain the correct pH for the precipitation of the calcium oxalate. It was found that using the amounts of sodium acetate called for by McCrudden, the solution was on the acid margin, and that larger quantities were preferable. The calcium was ignited and weighed in platinum crucibles as calcium oxide; the magnesium was precipitated as magnesium ammonium phosphate and weighed as pyrophosphate. The total phosphorus was determined in urine after ashing by Neumann's method (35) as directed by McCandless and Burton (36), precipitating as ammonium phosphomolybdate and magnesium ammonium phosphate and weighing as pyrophosphate. For all mineral determinations on foods and feces the ashed samples were used and the same methods employed as in urine. Ashing was done according to the Official Method (37), using an electric muffle furnace kept at approximately 450°C.

The micro Kjeldahl method of Koch and McMeekin (38) was employed for the nitrogen of the urine, and, with several modifications, for the food and feces. In order to get the two latter into solution and to work with weighable quantities, amounts containing from 30 to 100 mg. of nitrogen were digested for several hours in a small Kjeldahl flask with 50 cc. of concentrated sulfuric acid. To complete the oxidation several cc. of the 30 per cent hydrogen peroxide were then added in small portions, heating after each addition, the volume was made up to 100 cc. with distilled water, and 1 cc. used for Nesslerization.

The organic acids of the urine were determined daily by the Van Slyke and Palmer method (39); the creatinine, creatine, and ammonia by Folin's (40); and the hydrogen ion concentration by a modification of Henderson and Palmer's method described by Haskins (41). Qualitative tests for sugar, acetone, acetoacetic acids, and albumin were made daily and gave negative results in all cases.

All precautions of analytical work were used. Volumetric ware and weights were calibrated, blanks run on all materials, and each method was checked using chemicals of known composition. All analyses were done in duplicate or triplicate, usually in triplicate. For all nitrogen, organic acids, ammonia, and pH determinations fresh, doubly distilled water was used.

## RESULTS AND DISCUSSION.

The figures for the analyses of the food are given in Table II. For comparison, food composition figures from Sherman are also included, and the marked differences in the inorganic constituents, even more than in the nitrogen, illustrate well the necessity of analyzing the actual foods used in even approximate metabolism experiments rather than trusting to published figures. The results of the analyses of the urine and feces and the comparison with the intake are given in Tables III, IV, V, and VI.

The orange juice used in the second experiment was found by Miss Honora English to contain 0.98 per cent citric acid, 1.5 per cent total organic acid (by a modification of the Van Slyke and Palmer method for urine), and 1.03 per cent titratable acidity toward phenolphthalein.

The addition of orange juice to the diet of the children resulted in an increased retention by the body of calcium, phosphorus, magnesium, and nitrogen.

*Calcium Metabolism.*—The children both showed a slight positive calcium balance on the basal diet, but this was much increased in the orange juice period, chiefly through diminished excretion in the feces (Table III). The extra calcium retention in the orange juice period was considerably more than the little excess calcium in the diet of that period. For example, in Experiment II with D. B., when the intake of calcium was increased 0.20 gm. for the 3 days by the orange juice, an excess of 0.29 gm. was retained; and with L. F., 0.22 gm. additional calcium ingested resulted in 0.32 gm. extra retained. The percentage retained was also increased from 20.6 per cent without oranges, to 31.8 per cent with oranges in the case of D. B., and from 9.9 per cent to 23.2 per cent in the case of L. F.

The basal intake of calcium was kept at approximately that to which the children had been accustomed at the Home, and neither

TABLE II.

*Mineral and Nitrogen Content of Foods, in Percentage.**

| | Beef. | Potato. | Klim. | Bread. | Oat-meal. | Orange juice. |
|---|---|---|---|---|---|---|
| Calcium: | | | | | | |
| Experiment I. | | 0.0014 | | 0.024 | | |
| | | 0.0016 | 0.934 | 0.025 | | 0.0085 |
| " II. | | 0.0015 | | 0.061 | | |
| | | 0.0020 | 0.930 | 0.063 | | 0.0110 |
| Sherman's figures. | 0.013 | 0.014 | 0.960† | 0.027 | 0.069 | 0.029 |
| Phosphorus: | | | | | | |
| Experiment I. | 0.167 | 0.020 | | 0.102 | | |
| | 0.208 | 0.031 | 0.704 | 0.102 | 0.313 | 0.019 |
| " II. | 0.169 | 0.041 | | 0.097 | | |
| | 0.174 | 0.037 | 0.727 | 0.091 | 0.313 | 0.017 |
| Sherman's figures. | 0.243 | 0.058 | 0.744† | 0.093 | 0.392 | 0.016 |
| Magnesium: | | | | | | |
| Experiment I. | | 0.018 | | 0.030 | | |
| | | 0.025 | 0.096 | 0.029 | | 0.0137 |
| " II. | | 0.011 | | 0.042 | | |
| | | 0.012 | 0.093 | 0.049 | | 0.0130 |
| Sherman's figures. | 0.027 | 0.028 | 0.096† | 0.023 | 0.110 | 0.011 |
| Nitrogen: | | | | | | |
| Experiment I. | 3.66 | 0.33 | | 1.68 | | |
| | 3.44 | 0.25 | 4.25 | 1.64 | 2.44 | 0.123 |
| " II. | 3.22 | 0.39 | | 1.61 | | |
| | 3.23 | 0.42 | 4.25 | 1.67 | 2.65 | 0.148 |
| Sherman's figures. | 3.71 | 0.35 | 4.22† | 1.47 | 2.58 | 0.128 |

* Each percentage represents the average of two or three analyses of the food; when two percentages are given each represents the food used in a single period.

† This is eight times the value given for whole milk, since Klim diluted to eight times its weight is the equivalent of cow's whole milk.

it nor the slightly increased amount of the orange juice period was up to the optimum of 1 gm. per day recommended by Sherman and Hawley (25). It might, therefore, be thought that the increased retention with the orange juice was due to increased intake with its nearer approach to the optimum. However, the intake of the basal period in the second experiment was higher than that of the first, due to increased bread consumption, and yet the percentage retention was decidedly less and the variation in the actual retention irregular (with one child greater and the other less). Sherman and Hawley, also, when they increased the milk intake of their children, had an increase in actual amount of calcium retained, but, in many cases, according to calculations from their figures, a lowered percentage retained. Thus it seems to be demonstrated that some factor in the orange juice, beside the additional calcium, must have caused the retention of calcium.

TABLE III.

*Calcium Balance, Expressed in 3 Day Periods.*

| | D. B. | | | | L. F. | | | |
|---|---|---|---|---|---|---|---|---|
| | Period 1, without oranges. | | Period 2, with oranges. | | Period 1, without oranges. | | Period 2, with oranges. | |
| **Experiment I.** | | | | | | | | |
| | *gm.* | *per cent* | *gm.* | *per cent* | *gm.* | *per cent* | *gm.* | *per cent* |
| Intake | 1.19 | 100.0 | 1.33 | 100.0 | 1.17 | 100.0 | 1.33 | 100.0 |
| Feces | 0.72 | 60.6 | 0.70 | 53.0 | 0.72 | 61.0 | 0.76 | 57.5 |
| Urine | 0.16 | 13.1 | 0.21 | 14.8 | 0.13 | 11.4 | 0.16 | 11.7 |
| Total output | 0.88 | 73.7 | 0.91 | 67.8 | 0.85 | 72.4 | 0.92 | 69.2 |
| Retention | +0.31 | +26.3 | +0.42 | +32.2 | +0.32 | +27.6 | +0.41 | +30.8 |
| **Experiment II.** | | | | | | | | |
| Intake | 2.03 | 100.0 | 2.23 | 100.0 | 2.02 | 100.0 | 2.24 | 100.0 |
| Feces | 1.30 | 64.3 | 1.30 | 58.2 | 1.41 | 69.8 | 1.37 | 61.2 |
| Urine | 0.31 | 15.1 | 0.22 | 10.0 | 0.41 | 20.3 | 0.35 | 15.6 |
| Total output | 1.61 | 79.4 | 1.52 | 68.2 | 1.82 | 90.1 | 1.72 | 76.8 |
| Retention | +0.42 | +20.6 | +0.71 | +31.8 | +0.20 | +9.9 | +0.52 | +23.2 |

The results with phosphorus were similar to those with calcium (Table IV). In the first experiment the children had, during the ingestion of the basal diet, a slightly negative balance of 0.06 gm.

and 0.14 gm. phosphorus, but when the orange juice was added the balance became positive, 0.52 gm. and 0.57 gm. respectively. The results of the second experiment showed a slightly positive balance at the beginning and a decided increase in retention on the addition of oranges. As in all cases but one (Experiment II, L. F.) the additional amount of phosphorus retained on the ingestion of oranges was greater than the amount of phosphorus added by the oranges, it seems probable that here, as in the case

TABLE IV.

*Phosphorus Balance, Expressed in 3 Day Periods.*

| | D. B. | | | | L. F. | | | |
|---|---|---|---|---|---|---|---|---|
| | Period 1, without oranges. | | Period 2, with oranges. | | Period 1, without oranges. | | Period 2, with oranges. | |
| **Experiment I.** | | | | | | | | |
| | *gm.* | *per cent* | *gm.* | *per cent* | *gm.* | *per cent* | *gm.* | *per cent* |
| Intake | 2.61 | 100.0 | 2.91 | 100.0 | 2.56 | 100.0 | 2.91 | 100.0 |
| Feces | 0.80 | 30.7 | 0.72 | 24.8 | 0.72 | 28.2 | 0.55 | 19.6 |
| Urine | 1.87 | 71.5 | 1.66 | 57.1 | 1.98 | 77.3 | 1.79 | 61.6 |
| Total output | 2.67 | 102.2 | 2.38 | 81.9 | 2.70 | 105.5 | 2.34 | 81.2 |
| Retention | −0.06 | −2.2 | +0.53 | +18.1 | −0.14 | −5.5 | +0.57 | +18.8 |
| **Experiment II.** | | | | | | | | |
| Intake | 3.13 | 100.0 | 3.36 | 100.0 | 3.14 | 100.0 | 3.37 | 100.0 |
| Feces | 0.89 | 28.6 | 0.96 | 28.7 | 0.86 | 27.3 | 0.67 | 20.0 |
| Urine | 2.11 | 67.2 | 1.94 | 57.6 | 2.20 | 70.2 | 2.45 | 72.5 |
| Total output | 3.00 | 95.8 | 2.90 | 86.3 | 3.06 | 97.5 | 3.12 | 92.5 |
| Retention | +0.13 | +4.2 | +0.46 | +13.7 | +0.08 | +2.5 | +0.25 | +7.5 |

of calcium, there was some factor present which assisted the body in making an economical use of the mineral.

*Magnesium Metabolism.*—In spite of the slightly increased urinary and fecal output of magnesium during Period 2, the retention of magnesium by the body was increased (Table V), but this is less marked than is that of calcium and phosphorus, in which the body output was actually diminished when oranges were eaten.

*Nitrogen Metabolism.*—The intake of protein was made fairly high to relieve the monotony of the diet, approximately 13 per cent of the total calories in Experiment I, and 15 per cent in Experiment II, and in the latter experiment it was kept the same

during both periods, the additional nitrogen consumed in the orange juice being counterbalanced by a smaller bread consumption.

A marked retention of nitrogen resulted from the ingestion of the orange juice (Table VI). This is especially well demonstrated in the second experiment where with a constant nitrogen intake the nitrogen retained is increased by 2.05 gm. and 1.32 gm. in the 3 days when oranges are taken.

TABLE V.

*Magnesium Balance, Expressed in 3 Day Periods.*

| | D. B. | | | | L. F. | | | |
|---|---|---|---|---|---|---|---|---|
| | Period 1, without oranges. | | Period 2, with oranges. | | Period 1, without oranges. | | Period 2, with oranges. | |
| **Experiment I.** | | | | | | | | |
| | *gm.* | *per cent* | *gm.* | *per cent* | *gm.* | *per cent* | *gm.* | *per cent* |
| Intake | 0.68 | 100.0 | 0.87 | 100.0 | 0.66 | 100.0 | 0.86 | 100.0 |
| Feces | 0.25 | 37.5 | 0.26 | 30.2 | 0.16 | 23.7 | 0.22 | 25.0 |
| Urine | 0.28 | 40.5 | 0.33 | 38.0 | 0.21 | 32.0 | 0.31 | 36.4 |
| Total output | 0.53 | 78.0 | 0.59 | 68.2 | 0.37 | 55.7 | 0.53 | 61.4 |
| Retention | +0.15 | +22.0 | +0.28 | +31.8 | +0.29 | +44.3 | +0.33 | +38.6 |
| **Experiment II.** | | | | | | | | |
| Intake | 0.85 | 100.0 | 1.18 | 100.0 | 0.85 | 100.0 | 1.19 | 100.0 |
| Feces | 0.37 | 43.0 | 0.47 | 39.3 | 0.34 | 40.0 | 0.40 | 33.5 |
| Urine | 0.30 | 34.7 | 0.34 | 20.9 | 0.25 | 29.0 | 0.35 | 29.6 |
| Total output | 0.67 | 77.7 | 0.81 | 60.2 | 0.59 | 69.0 | 0.75 | 63.1 |
| Retention | +0.18 | +22.3 | +0.37 | +39.8 | +0.26 | +31.0 | +0.44 | +36.9 |

It is interesting to compare these results with the work of Yushiue (42) who found that avitaminosis was unfavorable to a positive nitrogen balance, even if a large amount of protein was fed, and also with the work of Borak (43) who secured less nitrogen retention on a predominantly acid-forming diet.

*Organic Acids, Ammonia, and Acidity.*—The addition of the orange juice to the diet increased the pH from 5.6 or 5.9, to 6.45, diminished the ammonia, and increased the organic acid. (Table VII gives the results for the second experiment. The first experiment showed no important differences from the second.) The

results of Blatherwick and Long (11) are thus confirmed and also those of McLaughlin and Blunt (44) who found increased urinary organic acids after eating other fruits. The increase of organic acid for the two children in the first experiment is equivalent to 6.1 per cent and 6.5 per cent of the 20.5 gm. of citric acid ingested in the orange juice, and in the second to 7.5 per cent and 7.4 per cent of the 21.6 gm. ingested.

*Creatinine and Creatine.*—These two substances were determined as a step in the correct determination of the organic acids (Table

TABLE VI.

*Nitrogen Balance, Expressed in 3 Day Periods.*

| | D. B. | | | | L. F. | | | |
|---|---|---|---|---|---|---|---|---|
| | Period 1, without oranges. | | Period 2, with oranges. | | Period 1, without oranges. | | Period 2, with oranges. | |
| **Experiment I.** | | | | | | | | |
| | *gm.* | *per cent* | *gm.* | *per cent* | *gm.* | *per cent* | *gm.* | *per cent* |
| Intake | 31.58 | 100.0 | 34.48 | 100.0 | 30.98 | 100.0 | 34.53 | 100.0 |
| Feces | 2.46 | 7.8 | 2.54 | 7.4 | 3.83 | 12.4 | 3.47 | 10.0 |
| Urine | 28.22 | 89.0 | 27.86 | 80.8 | 25.31 | 81.7 | 24.96 | 72.3 |
| Total output | 30.68 | 96.8 | 30.40 | 88.2 | 29.14 | 94.1 | 28.43 | 82.3 |
| Retention | +0.90 | +3.2 | +4.08 | +11.8 | +1.84 | +5.9 | +6.10 | +17.7 |
| **Experiment II.** | | | | | | | | |
| Intake | 41.20 | 100.0 | 41.22 | 100.0 | 41.29 | 100.0 | 41.42 | 100.0 |
| Feces | 2.44 | 5.9 | 3.89 | 9.4 | 4.06 | 9.8 | 3.65 | 8.8 |
| Urine | 32.00 | 77.7 | 28.52 | 69.2 | 30.81 | 74.6 | 30.03 | 72.5 |
| Total output | 34.44 | 83.6 | 32.41 | 78.6 | 34.87 | 84.4 | 33.68 | 81.3 |
| Retention | +6.76 | +16.4 | +8.81 | +21.4 | +6.42 | +15.6 | +7.74 | +18.7 |

VII). The creatine excretion of the girls was remarkably high, several times half of the total creatinine. Apparently it was uninfluenced by the orange juice.

*Gain in Weight during the Experiments.*—When orange juice was added to the diet both children showed marked gain in weight (Table VIII). During the ingestion of the basal diet there was a much better gain than that obtained while at the Home and greater than that expected for children of their ages, an improvement which may be attributed to the quieter life led by the girls and

especially to the larger food intake; but on the addition of oranges, the gain in weight was three to four times as much daily as in the period when oranges were not included in the menu.

This is hardly explainable merely on the basis of the additional calories ingested—about 300 more when oranges were added to

TABLE VII.

*Total Acidity, Ammonia, Organic Acids, Creatinine, and Creatine in the 24 Hour Urine.*

*Experiment II.*

| | Volume. | pH | Ammonia nitrogen. | Organic acid 0.1 N. | Preformed creatinine. | Creatine. |
|---|---|---|---|---|---|---|
| | *cc.* | | *mg.* | *cc.* | *mg.* | *mg.* |
| D. B., Period 1: | | | | | | |
| Day 1 | 940 | 5.9 | 523 | 290 | 793 | 331 |
| " 2 | 1250 | 5.9 | 549 | 161 | 704 | 407 |
| " 3 | 1040 | 5.9 | 474 | 229 | 739 | 332 |
| Average | ..... | ..... | 515 | 227 | 745 | 357 |
| Period 2: | | | | | | |
| Day 1 | 1360 | 6.45 | 446 | 287 | 721 | 446 |
| " 2 | 1268 | 6.45 | 446 | 336 | 867 | 446 |
| " 3 | 1390 | 6.45 | 386 | 308 | 758 | 386 |
| Average | ..... | ..... | 426 | 310 | 782 | 426 |
| L. F., Period 1: | | | | | | |
| Day 1 | 1240 | 5.9 | 500 | 182 | 730 | 402 |
| " 2 | 1235 | 5.9 | 500 | 218 | 632 | 534 |
| " 3 | 935 | 5.9 | 500 | 238 | 666 | 503 |
| Average | ..... | ..... | 500 | 213 | 676 | 480 |
| Period 2: | | | | | | |
| Day 1 | 1210 | 6.45 | 403 | 288 | 646 | 492 |
| " 2 | 1215 | 6.45 | 369 | 282 | 769 | 323 |
| " 3 | 1426 | 6.45 | 414 | 319 | 678 | 411 |
| Average | ..... | | 395 | 296 | 698 | 409 |

the diet—for an even larger increase in calories from the Home diet to that of the basal diet of the experiment did not cause the marked stimulation in growth. In Experiment II in the 9 days of the orange period D. B. gained 2.9 pounds as against 1.2 pounds in the 14 days of the basal diet, or figured per day, 3.7 times as much in

the orange period. L. F.'s gain increased from 1.2 pounds in the basal diet period to 3.3 pounds when oranges were eaten, being 4.3 times as rapid when oranges were fed.

How the orange brings about the favorable nitrogen and mineral retention is unknown. The effect may be due to one or several of the following factors—the vitamins which promote the economical use of elements already present but not efficiently used; the additional calcium and phosphorus which induces the retention of these and possibly other elements in excess of the amount added; some factor which stimulates a greater flow of hydrochloric acid in

TABLE VIII.

*Gain in Weight as Influenced by Orange Feeding.*

| Experiment. | Subject. | Length of period. | First weight. | Last weight. | Gain in weight per day. |
|---|---|---|---|---|---|
| | | Period 1, without oranges. | | | |
| | | *days* | *lbs.* | *lbs.* | *lb.* |
| I | D. B. | 5 | 65.6 | 66.2 | 0.12 |
| I | L. F. | 5 | 61.2 | 61.7 | 0.10 |
| II | D. B. | 14 | 66.6 | 67.8 | 0.085 |
| II | L. F. | 14 | 61.1 | 62.3 | 0.085 |
| | | Period 2, with oranges. | | | |
| I | D. B. | 5 | 66.2 | 67.1 | 0.18 |
| I | L. F. | 5 | 61.7 | 63.4 | 0.34 |
| II | D. B. | 9 | 67.8 | 70.7 | 0.32 |
| II | L. F. | 9 | 62.3 | 65.6 | 0.37 |

the stomach, causing a greater acidity in the upper part of the small intestine and a greater absorption of minerals; or the basic residue which may favor normal activities in the growing body. Work on the solution of these problems is planned.

## SUMMARY.

A study was conducted on two growing girls, 10 and 11 years old, to determine the effect of orange juice on calcium, phosphorus, magnesium, and nitrogen metabolism.

The basal diet consisting of oatmeal, rice, flour, bread, potato,

sugar, oleomargarine fat, lean beef, and whole milk powder was ingested for a preliminary adjustment period and a 3 day collection period. Then orange juice, 600 to 700 cc. daily, was added to the diet; another adjustment period and collection period followed. After an interval of 2½ months the experiment was again performed on the same girls.

Calcium assimilation was decidedly benefited when oranges formed a part of the diet, the increased retention being considerably greater than the calcium added in the oranges and greater than might be expected from a stimulus to retention caused by a larger calcium intake.

The increase in phosphorus retention was even more marked than that of calcium, more than three times as much phosphorus, both in gm. and percentage of intake, being assimilated when orange juice was added.

The magnesium retention was also increased, although to a less marked extent than that of calcium and phosphorus.

Nitrogen assimilation was greater when orange juice was ingested, even though the nitrogen intake was not altered.

Urinary ammonia was decreased, and urinary pH and organic acids increased, thus confirming the work of Blatherwick and Long. The increase of organic acids amounted to approximately 7 per cent of the citric acid of the orange juice ingested.

A marked increase in the children's weight was observed.

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# MAINTENANCE VALUES FOR THE PROTEINS OF MILK, MEAT, BREAD AND MILK, AND SOY BEAN CURD.

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The recent exhaustive review by Mitchell (1) of the literature relating to the nutritive value of proteins renders superfluous any extensive discussion of this topic by us. Consequently we shall review very briefly only such papers as have to do with the utilization by the human subject of the foods under immediate consideration.

Beyond doubt the search for an absolute biological constant in protein metabolism is a vain one; any so called biological minimum is such only for a given set of conditions. The influence of the energy value of the diet upon the efficiency with which its protein is utilized has long been recognized. Neumann's (2) 40 day experiment upon himself is an excellent illustration. Living exclusively upon rye bread, cane sugar, and water, he found in a 5 day period on bread alone, with 42.3 calories per kilo and total nitrogen intake 9.9 gm. daily, a negative nitrogen balance; in the next 5 day period the addition of 300 gm. of cane sugar (making 63.8 calories per kilo) changed this to a positive balance; and in the succeeding 8 day period with 500 gm. of cane sugar (making 77.1 calories per kilo) there was storage of 1.6 gm. of nitrogen daily; in other words, increasing the calories 55 per cent changed the nitrogen situation from a loss equal to 20 per cent of the intake to a storage equal to 17 per cent.

Cathcart (3) has lately called attention to the difference of opinion existing in regard to the influence of muscular work upon the nitrogen output, and cites about twice as many investigators

in support of the idea that work increases protein metabolism as against it. It seems important, therefore, in comparing experiments to determine nitrogen minimum, to see that there is no marked difference in the amount of muscular activity.

Furthermore, the influence of previous diet and the power of the body to make adaptation to different levels of nitrogen intake demand that in experiments to determine biological efficiency there be relatively long periods in which all conditions of living, including diet, are kept as uniform as possible.

Thomas (4) attempted to determine biological values for a considerable number of foods and these seem to have been quite widely accepted without consideration of the experimental conditions. Mitchell (1) has very justly criticized the conditions under which Thomas' work was done.

Thomas (4) assumes that any nitrogen in the feces in excess of an average figure on a nitrogen-free diet is to be charged against the food as cost of digestion or unavailable nitrogen. With this general assumption most are agreed. But when one examines the data as to nitrogen excretion on a diet as near nitrogen-free as the experimenters could get it one finds that in those cases (collected and tabulated by Thomas) where the nitrogen intake was less than 1 gm. per day, the fecal nitrogen ranged from 0.54 to 1.52 gm. per day, and that in a total of 48 cases in which nitrogen intake did not exceed about 3 gm. per day the range of nitrogen in the feces is from 0.22 to 1.75 gm.! Since the mean of these is 0.99, Thomas assumes an average of 1 gm. per day as representing metabolic nitrogen in feces for himself, although his own two periods, one of 2 days, the other of 4 days, on a nitrogen-free diet show 0.97 and 0.45 gm. per day respectively, and a third period of 3 days in which the average was 2.99 is disregarded entirely. With so much variation, a long time would certainly be required to establish a satisfactory figure for fecal metabolic nitrogen. In the experiments on wheat, the fecal nitrogen was noticeably low, and its probable relation to the diet as a whole rather than the protein alone is shown by the following protocol.

| Date. | Nitrogen intake. | Balance. | Calories. | | Nitrogen in feces. |
|---|---|---|---|---|---|
| | | | Total. | Per kilo. | |
| | *gm.* | *gm.* | | | *gm.* |
| Mar. 23 | 13.48 | −4.89 | ? | ? | 0.51 |
| " 24 | 12.60 | −5.79 | 1411 | 20 | 0.50 |
| " 25 | 13.60 | +1.05 | 2722 | 37 | 0 96 |

On no day were conditions suitable for determining the maximum efficiency of the protein, since the total calories were very low for 2 days, and the effect of nearly doubling them, while marked, would not be fully

shown in a single following day. But the nitrogen in the feces at its highest is as low as the average Thomas assumes for fecal metabolic nitrogen. It is evident that with no more calories he could have attained nitrogen equilibrium on much less nitrogen than he actually took. Sherman (5), in his experiments with E. O. on white bread, secured nitrogen equilibrium with about 35 calories per kilo and less than half as much nitrogen per kilo as Thomas. It is interesting to note that E. O's fecal nitrogen for 12 days averaged 1.0 gm. per day and was practically uniform in all periods. Thomas' one day experiment on cow's milk is not comparable with that on bread, as the calories were much higher and the nitrogen intake much lower. Although the fecal nitrogen was more than 20 per cent of the nitrogen intake or about three times as much as on the bread diet, the use of an arbitrary formula makes the bread appear less than half as efficient as the milk, a conclusion not at all justified by the data.

Martin and Robison (6) have reported a most carefully planned experiment in which, in a 7 day period on a diet nearly nitrogen-free and yielding from 45 to 50 calories per kilo, they reduced their nitrogen output to 0.052 and 0.049 gm. per kilo respectively. They then attempted to secure nitrogen equilibrium when this amount of nitrogen was derived from milk. These authors were also too hasty in changing calories or protein or both. Equilibrium was almost reached by Martin in a 12 day experiment in which the diet yielded 53 calories and 0.085 gm. of nitrogen per kilo, and by Robison in a 5 day experiment with 48 calories and 0.052 gm. of nitrogen per kilo. Martin was thin and took the diet with little appetite and more or less digestive disturbance, the feces being frequently more or less fluid. Nevertheless, there was a slight gain in body weight during the experiment.

Less consistently planned and much less fully reported are somewhat similar experiments by Lauter and Jenke (7), who also gave a nitrogen-free diet to establish the subject's nitrogen minimum. They then fed sometimes as much nitrogen from the food under investigation as the total nitrogen output, and sometimes only the equivalent of the urinary nitrogen. There were various subjects for the different foods, and no data are given in regard to the energy value of the diets, so that it is impossible to tell how far they are comparable with each other or with those of other investigators. In the most satisfactory experiment on meat, a man weighing 58 kilos reached in 10 days on a nitrogen-free diet a daily output of 3.5 gm. (urine 2.2 gm., feces 1.3 gm.); for 4 days thereafter he took daily 3.4 gm. of nitrogen in the form of meat, with an average nitrogen excretion of 3.85 gm. (urine 2.6 gm., feces 1.25 gm.). Since the fecal nitrogen was practically the same in both cases, it would appear, granting that the diets had the same fuel value, that equilibrium on meat required an increase of 8.5 per cent above the output on a nitrogen-free diet. The data in the experiments with wheat flour are not sufficiently complete to make a comparison between the meat and wheat, but appear to indicate that the wheat was somewhat less efficiently utilized than the meat, and this difference was due to an increase in urinary rather than fecal nitrogen.

If 100 per cent biological efficiency means the attainment of nitrogen equilibrium on the same amount of nitrogen as is lost from the body on a nitrogen-free diet ample in calories, there is as yet no evidence that any food will score 100. But by keeping calories constant and protein at a level which should theoretically just meet requirement, it is possible to compare two kinds of protein as to their biological efficiency. Given perfect digestion and uniform experimental conditions over a considerable period, the amount of nitrogen in the feces should be fairly constant for a given food. Sherman (5) has shown that the minimum protein requirement for maintenance in adults averages 44.4 ± 0.6 gm. per 70 kilo or between 0.096 and 0.107 gm. of nitrogen per kilo of body weight, and has also shown that the protein of rations consisting essentially of corn-meal or oatmeal supplemented by very small amounts of milk (not over 0.1 to 0.2 of the food protein) may be fully as efficient as the protein of ordinary mixed diets. Data on these experiments are included in Table I, in which have been brought together the experiments most closely related to the work which we are reporting. Rubner (8), during the war, carried on extensive investigations as to the comparative value of protein from certain vegetable foods and various kinds of rye bread, among which are three studies, each of 6 weeks duration, on "fine white bread;" but as the nitrogen intake averaged over 12 gm. per man per day, and in the one case where body weight was stated amounted to 0.2 gm. per kilo, these have no direct bearing on the question of maintenance values.

## EXPERIMENTAL.

Eight experiments, each lasting from 12 to 15 days, have been conducted on four healthy young women. All living conditions were much the same, as these were students working in the nutrition laboratory. The water drunk was kept uniform for each subject. For several days prior to the collection of samples for analysis the subjects subsisted on the diet to be investigated, thus getting somewhat adjusted to the experimental diet. The basal diet consisted of arrowroot starch, Dextri-Maltose, lactose, clarified butter fat and apple. Small thin biscuit were made from the arrowroot starch, Dextri-Maltose, and part of the lactose with the aid of water and a very little baking powder. The apple was cooked with the lactose and eaten as apple sauce. The butter fat was prepared by melting the butter at a low temperature, decanting and filtering through a thick layer of absorbent cotton, so that a perfectly clear product was obtained. The

TABLE I.

*Comparable Experiments.*

| Author. | Subject. | Food studied. | Total N from food studied. | No. of days. | Body weight. | Calories per kilo. | Nitrogen intake. | | Nitrogen output. | Balance. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Total. | Per kilo. | | |
| | | | *per cent* | | *kg.* | | *gm.* | *gm.* | *gm.* | *gm.* |
| Sherman (5). | E. O. | White bread. | 100 | 12 | 80.0 | 34.7 | 6.00 | 0.076 | 6.08 | −0.08 |
| Abderhalden and others (9). | H. R. | White bread. | 100 | 5 | 61.6 | 56.0 | 8.17 | 0.133 | 8.30 | −0.13 |
| | | | 100 | 5 | 62.1 | 57.0 | 9.12 | 0.147 | 9.44 | −0.32 |
| Sherman and Winters (10). | J. W. | Corn-meal. | 97 | 8 | 54.9 | 36.0 | 4.26 | 0.089 | 4.89 | −0.63 |
| | " | " | 97 | 16 | 54.4 | 37.0 | 4.36 | 0.090 | 4.82 | −0.46 |
| | " | Corn-meal and milk. | 73 / 25 } 98 | 28 | 54.6 | 40.0 | 5.85 | 0.106 | 5.72 | +0.13 |
| | " | " " " | 88 / 10 } 98 | 16 | 54.0 | 39.0 | 4.83 | 0.087 | 4.71 | +0.15 |
| Sherman, Winters, and Phillips (11). | P. | Oatmeal. | 95 | 12 | 67.0 | 38.0 | 5.30 | 0.090 | 6.00 | −0.70 |
| | " | Oatmeal and milk. | 87 / 8 } 95 | 12 | 67.0 | 38.0 | 5.80 | 0.092 | 6.20 | −0.40 |
| Martin and Robison (6). | M. | Milk. | 96 | Last 4 of 5 | 62.2 | 44.0 | 4.17 | 0.067 | 5.58 | −1.41 |
| | " | " | 98 | Last 6 of 12 | 62.0 | 53.0 | 5.25 | 0.085 | 5.61 | −0.36 |
| | " | " | 98 | Last 4 of 8 | 62.2 | 48.0 | 6.35 | 0.102 | 7.22 | −0.87 |
| | " | " | 99 | Last 6 of 10 | 61.7 | 46.0 | 11.44 | 0.185 | 11.23 | +0.21 |
| | R. | " | 90 | Last 4 of 5 | 57.5 | 48.0 | 2.98 | 0.052 | 3.63 | −0.65 |
| | " | " | 98 | Last 4 of 7 | 57.9 | 48.0 | 6.29 | 0.110 | 7.73 | −1.44 |

meat was lean round of beef, freed from all visible fat and connective tissue, ground in a chopper and made into small cakes for broiling. The white bread was a standard market variety. The soy bean curd was made in the Chinese manner by Miss Yen. In the meat periods sufficient calcium lactate was taken to secure the same calcium content for the diet as in the milk periods. The energy value of the diets was kept uniform for all subjects at 40 calories per kilo of body weight. This was possible as they were all indulging in about the same amount of muscular activity. Not more than 3 per cent of the protein in any diet came from foods other than the one under investigation. The basal diet proved very easy to digest and the few occasions of slight indisposition which occurred were due to

TABLE II.

*Kinds and Amounts of Food Eaten.*

| No. of experiment | I | II | III | IV | V | VI | VII | VIII |
|---|---|---|---|---|---|---|---|---|
| Subject | A | A | A | B | B | C | C | D |
| Body weight, *kg.* | 54 | 54 | 54 | 62.8 | 62.8 | 69.7 | 69.7 | 50.4 |
| Energy value of diet, *cal. per kg.* | 40 | 40 | 40 | 41.5 | 41.5 | 37.5 | 37.5 | 40 |
| | Weight of food. | | | | | | | |
| | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* |
| Arrowfoot starch | 125 | 125 | 73 | 138 | 138 | 144 | 144 | 150 |
| Dextri-Maltose | 45 | 45 | 45 | 50 | 50 | 52 | 52 | 45 |
| Lactose | 125 | 85 | 100 | 94* | 188 | 98 | 144 | 75 |
| Butter fat | 75 | 53 | 67 | 58 | 83 | 61 | 86 | 73 |
| Apple | 200 | 200 | 200 | 220 | 220 | 230 | 230 | 230 |
| Milk | | 794 | 397 | 873† | | 913‡ | | |
| Bread | | | 141 | | | | | |
| Meat | 124 | | | | 145 | | 159 | |
| Soy bean curd | | | | | | | | 246 |
| Calcium lactate | 3.8 | | | | 4.5 | | 5 | |

* Increased after 11 days to 144 gm.
† " " 6 " " 934 "
‡ " " 6 " " 1024 "

fatigue rather than to the diet.[1] Quantitative data on the diets are given in Table II and the source and amount of nitrogen in each diet in Table III.

Subject A began with the meat diet and after a period of 15 days shifted to the milk diet for 12 days and finally to the bread and milk diet for 12 days, making 39 consecutive days in which the nitrogen intake did not exceed 0.077 gm. per kilo of body weight. Subjects B and C began with the milk rather than the meat diet, to see whether there would be any difference

[1] A preliminary report embracing part of this work was published by Rose, M. S., MacLeod, G., and Bisbey, B., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 143.

TABLE III.

*Sources and Amounts of Nitrogen in Each Diet.*

| Experiment No. | I | | II | | III | | IV | | V | | VI | | VII | | VIII | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Subject | A | | A | | A | | B | | B | | C | | C | | D | |
| Daily intake of nitrogen | gm. | per cent | gm. | per cent | gm. | per cent | gm. | per cent | gm. | per cent | gm. | per cent | gm. | per cent | gm. | per cent |
| Milk | | | 4.05 | 97.6 | 2.06 | 50.0 | 4.67 | 97.0 | | | 5.10 | 97.0 | | | | |
| Bread | | | | | 1.97 | 48.0 | | | | | | | | | | |
| Meat | 4.22 | 97.7 | | | | | | | 4.92 | 97.0 | | | 5.39 | 97.0 | | |
| Soy bean curd | | | | | | | | | | | | | | | 3.90 | 97.5 |
| Apple | 0.07 | 1.6 | 0.07 | 1.7 | 0.07 | 1.7 | 0.10 | 2.0 | 0.10 | 2.0 | 0.11 | 2.0 | 0.11 | 2.0 | 0.07 | 1.8 |
| Dextri-Maltose | 0.03 | 0.7 | 0.03 | 0.7 | 0.02 | 0.3 | 0.05 | 1.0 | 0.05 | 1.0 | 0.05 | 1.0 | 0.05 | 1.0 | 0.03 | 0.7 |
| Total nitrogen per day | 4.32 | | 4.15 | | 4.12 | | 4.82 | | 5.07 | | 5.26 | | 5.55 | | 4.00 | |
| Nitrogen per kilo of body weight | 0.080 | | 0.077 | | 0.076 | | 0.077 | | 0.081 | | 0.076 | | 0.080 | | 0.080 | |

in shifting from meat to milk or *vice versa*. The daily intake and output of nitrogen are summarized in Table IV and a comparison of results on the basis of body weight in Table V. The protocols are given in Table VIII.

The nitrogen intake ranged from 0.075 to 0.081 gm. per kilo per day, which is 80 to 85 per cent of Sherman's maintenance average, and no subject showed a negative balance in the final average of any experiment. The output of nitrogen in the urine averages 0.062 gm. per kilo for the milk diet, and 0.069 gm. for the meat diet, indicating a slightly better utilization of the milk than the meat. The one experiment on bread and milk shows

TABLE IV.

*Summary of Daily Intake and Output of Nitrogen (for Protocols see Table VIII.)*

| Experiment No. | Food. | No. of days. | Subject. | Body weight. | Nitrogen. | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Food. | Urine. | Feces. | Output. | Balance. |
| | | | | *kg.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* |
| II | Milk. | 12 | A | 54.0 | 4.15 | 3.23 | 0.37 | 3.60 | +0.55 |
| IV | " | Last 9 of 12 day period. | B | 62.7 | 4.82 | 4.15 | 0.62 | 4.77 | +0.05 |
| VI | " | " " " | C | 69.6 | 5.25 | 4.16 | 0.63 | 4.79 | +0.46 |
| I | Meat. | 15 | A | 54.0 | 4.32 | 3.87 | 0.39 | 4.26 | +0.06 |
| V | " | 12 | B | 62.6 | 5.06 | 4.31 | 0.67 | 4.98 | +0.08 |
| VII | " | 12 | C | 69.7 | 5.54 | 4.75 | 0.79 | 5.54 | ±0.00 |
| III | Milk and bread. | 12 | A | 54.0 | 4.12 | 3.22 | 0.50 | 3.72 | +0.40 |
| VIII | Soy bean curd. | 12 | D | 50.4 | 4.00 | 3.00 | 0.97 | 3.97 | +0.13 |

practically the same urinary nitrogen as the milk alone. The fecal nitrogen was remarkably uniform for all subjects on the milk, meat, and bread and milk diets, averaging 11.3 per cent of the total intake on the milk, 11.9 per cent on the meat, and 12.1 per cent on the bread and milk diet. On the soy bean diet there is a striking difference, the fecal nitrogen being 24 per cent of the intake. In view of this high fecal output it is surprising that the subject should have maintained equilibrium with as low a nitrogen intake as the other subjects. Studies of the nitrogen metabolism of the Chinese (12) have given results showing the quantities of

nitrogen and urea excreted in the urine of the Chinese to be low as compared with those obtained for Europeans.

Subject A on the three diets, milk, meat, and bread and milk, differing in nitrogen content by less than 4 mg. per kilo, was able to store daily nine times as much on milk as on meat and seven times as much on bread and milk as on meat. In other words, the milk was used with greater economy than the meat and the

TABLE V.

*Comparison of Results on the Basis of 1 Kilo of Body Weight.*

| Diet. | Subject. | Experiment No. | Nitrogen per kilo of body weight. | | | | | Nitrogen intake.* | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Intake. | Total output. | Urine. | Feces. | Balance. | In feces. | Stored. |
| | | | gm. | gm. | gm. | gm. | gm. | per cent | per cent |
| Milk | A | II | 0.0769 | 0.0667 | 0.060 | 0.007 | +0.0102 | 8.9 | 13.3 |
| " | B | IV | 0.0767 | 0.0760 | 0.066 | 0.010 | +0.0007 | 12.9 | 0.9 |
| " | C | VI | 0.0754 | 0.0688 | 0.060 | 0.009 | +0.0066 | 12.0 | 8.7 |
| Average | | | 0.0763 | 0.0705 | 0.062 | 0.009 | +0.0058 | 11.3 | 7.6 |
| Meat | A | I | 0.0800 | 0.0789 | 0.072 | 0.007 | +0.0011 | 9.0 | 1.4 |
| " | B | V | 0.0810 | 0.0798 | 0.069 | 0.011 | +0.0012 | 13.2 | 1.6 |
| " | C | VII | 0.0795 | 0.0794 | 0.068 | 0.011 | +0.0001 | 14.3 | 0.1 |
| Average | | | 0.0801 | 0.0793 | 0.069 | 0.010 | +0.0008 | 12.2 | 1.0 |
| Bread and milk | A | III | 0.0763 | 0.0689 | 0.060 | 0.009 | +0.0074 | 12.1 | 9.1 |
| Soy bean curd | D | VIII | 0.0794 | 0.0788 | 0.060 | 0.019 | +0.0006 | 24.2 | 3.3 |

* Calculated on the daily average for the period.

bread and milk with practically the same efficiency as the milk alone, which is what we might expect in view of Sherman's results with bread alone (5). Subject B was just about in equilibrium on both milk and meat diets. On so small a margin, the differences between the two diets have probably little significance. Subject C was just about in equilibrium on the meat diet, but was able to store 8.7 per cent of the intake on the milk diet. The soy bean diet needs further investigation with other subjects.

By reference to Table V it will be seen that the distribution of nitrogen between urine and feces on the milk, meat, and bread and milk diets was strikingly similar in all cases. There is no reason to think that the "cost of digestion" is greater for one of these diets than the others. On the soy bean diet, the distribution was quite different and corresponds with Neumann's work on rye bread, where, in his best balance experiment, approximately 25 per cent of the ingested nitrogen was eliminated as fecal nitrogen: and also with Martin and Robison's work on whole wheat bread, where the fecal nitrogen was from 14 to 29 per cent of the total intake. Further experiments are needed to establish nitrogen minima on diets of this type as compared with white bread, milk, meat, and bread and milk. The experience of Sherman and his coworkers (see Table I) with corn-meal and oats would indicate that from 12 to 17 per cent more nitrogen was needed to secure equlibrium on these foods, even with a little milk in the diet, than is required with white bread, milk, meat, or bread and milk.

In these experiments the acid-base balance of the milk and meat diets was similar, in spite of the potential acidity of the meat, owing to the addition of calcium lactate to the meat diet. Abderhalden and his associates (9) observed in their experiments to determine protein minimum on potatoes and on bread, that the amount of urinary nitrogen increased upon changing from the potato to the bread diet, and attributed the difference to the greater potential acidity of the bread. McCollum and Hoagland (13) found additional nitrogen in the form of ammonia eliminated on an acid as compared with an alkaline diet in the case of the pig. We hoped, by equalizing the calcium in the milk and meat diets, to rule out any influence of the acid-base balance upon the results. To test the point, the urine of subjects B and C was analyzed for acidity, ammonia, and also for creatinine throughout the 25 days of their experiments. Total acidity of the urine was determined by Folin's method, hydrogen ion concentration with a potentiometer, creatinine with a Duboscq colorimeter, and ammonia by Folin's aspiration method. Acidity was determined for 1 day in the middle of each experiment, but ammonia and creatinine daily. The acid-base balance of the diet was estimated

TABLE VI.

*Acid-Base Balance of Diets and Acidity of Urine.*

| | Acid-base balance of diet.* | | | | Urine.† | |
|---|---|---|---|---|---|---|
| | Food. | Calories. | Excess acid (n/1 NaOH). | Excess base (n/1 HCl). | Total acidity (n/10 NaOH). | pH |
| **Milk diet.** | | | | | | |
| | | | cc. | cc. | cc. | |
| Subject B, Experiment IV. | Apple. | 139 | | 8.34 | | |
| | Milk. | 645 | | 16.77 | | |
| Total | | | | 25.11 | 273.8 | 6.20 |
| Subject C, Experiment VI. | Apple. | 145 | | 8.70 | | |
| | Milk. | 707 | | 18.38 | | |
| Total | | | | 27.08 | 230.0 | 7.85 |
| **Meat diet.** | | | | | | |
| Subject B, Experiment V. | Apple. | 139 | | 8.34 | | |
| | Meat. | 226 | 22.6 | | | |
| | Calcium lactate 4.5 gm. | | | 29.20 | | |
| Total | | | 22.6 | 37.54 | 257.2 | 6.15 |
| Excess base | | | | 14.94 | | |
| Subject C, Experiment VII. | Apple. | 145 | | 8.70 | | |
| | Meat. | 248 | 24.8 | | | |
| | Calcium lactate 5 gm. | | | 32.46 | | |
| Total | | | 24.8 | 41.16 | 125 | 6.97 |
| Excess base | | | | 16.36 | | |

* Calculated from Sherman, H. C., Food products, New York, 1914.

† Determined for 1 day at middle of each experiment.

from data given by Sherman. The data are given in Tables VI and VII.

Both subjects had about the same excess of base in their milk

TABLE VII.

*Comparison of Output of Ammonia and Creatinine on Milk and Meat Diets.*

| Date. | Subject B, Experiment IV. Volume of urine. | Ammonia. | Creatinine. | Subject C, Experiment VI. Volume of urine. | Ammonia. | Creatinine. |
|---|---|---|---|---|---|---|
| **I. Milk diet.** | | | | | | |
| | *cc.* | *gm.* | *gm.* | *cc.* | *gm.* | *gm.* |
| Mar. 15 | 1680 | 0.36 | 1.31 | 1600 | 0.29 | 1.04 |
| " 16 | 1520 | 0.39 | 1.28 | 1560 | 0.31 | 1.09 |
| " 17 | 1640 | 0.26 | 1.19 | 1200 | 0.28 | 0.92 |
| " 18 | 1660 | 0.28 | 1.32 | 1420 | 0.34 | 1.03 |
| " 19 | 1660 | 0.38 | 1.32 | 1640 | 0.34 | 1.93 |
| " 20 | 1160 | 0.29 | 1.30 | 1250 | 0.29 | 0.94 |
| " 21 | 1540 | 0.40 | 1.35 | 1560 | 0.29 | 1.01 |
| " 22 | 1420 | 0.44 | 1.31 | 1600 | 0.35 | 1.03 |
| " 23 | 1400 | 0.32 | 1.35 | 1045 | 0.31 | 1.12 |
| " 24 | 1540 | 0.32 | 1.31 | 1260 | 0.27 | 1.07 |
| " 25 | 1680 | 0.33 | 1.38 | 1225 | 0.38 | 1.00 |
| " 26 | 1310 | 0.31 | 1.42 | 1530 | 0.36 | 0.92 |
| Average..... | | 0.34 | 1.32 | | 0.32 | 1.09 |
| **II. Meat diet.** | | | | | | |
| | Subject B, Experiment V. | | | Subject C, Experiment VII. | | |
| Mar. 27 | 1390 | 0.31 | 1.49 | 1150 | 0.33 | 1.13 |
| " 28 | 1215 | 0.32 | | 1530 | 0.27 | 1.11 |
| " 29 | 1060 | 0.36 | 1.40 | 1510 | | |
| " 30 | 1950 | 0.33 | 1.28 | 1800 | 0.30 | 1.22 |
| " 31 | 970* | | | 1150 | 0.27 | 0.98 |
| Apr. 1 | 1320 | 0.41 | 1.42 | 1640 | 0.30 | 1.16 |
| " 2 | 1220 | 0.48 | 1.28 | 1010 | 0.41 | 1.21 |
| " 3 | 1310 | 0.41 | 1.71 | 1155 | 0.50 | 1.17 |
| " 4 | 1440 | 0.39 | 1.33 | 1400 | 0.42 | 1.17 |
| " 5 | 2000 | 0.59 | 1.38 | 1480 | 0.38 | 1.07 |
| " 6 | 1670 | 0.49 | 1.50 | 1560 | 0.41 | 1.22 |
| " 7 | 1980 | 0.39 | 1.42 | 1980 | 0.43 | 1.19 |
| Average..... | | 0.41 | 1.42 | | 0.37 | 1.15 |

* Part of sample lost (about 300 cc.).

diets (equivalent to 25 and 27 cc. of normal acid) and showed no marked difference in the total acidity of the urine, though the hydrogen ion concentration was a little higher for subject B than

TABLE VIII.

*Appendix.—Protocols of Nitrogen Balance Experiments.*

| Period. | Date. | Body weight. | Nitrogen. | | | | |
|---|---|---|---|---|---|---|---|
| | | | In food. | In urine. | In feces. | Output. | Balance. |
| Daily intake and output of nitrogen in Experiment I. Subject A., meat diet. | | | | | | | |
| | | *kg.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* |
| I | Mar. 3 | | 4.32 | 3.94 | | | |
| | " 4 | | 4.32 | 4.09 | | | |
| | " 5 | | 4.32 | 3.72 | | | |
| Average..... | | 54.0 | 4.32 | 3.92 | 0.29 | 4.21 | +0.11 |
| II | Mar. 6 | | 4.32 | 4.01 | | | |
| | " 7 | | 4.32 | 3.88 | | | |
| | " 8 | | 4.32 | 3.86 | | | |
| Average..... | | 54.0 | 4.32 | 3.92 | 0.42 | 4.34 | −0.02 |
| III | Mar. 9 | | 4.32 | 4.00 | | | |
| | " 10 | | 4.32 | 4.10 | | | |
| | " 11 | | 4.32 | 4.20 | | | |
| Average..... | | 54.0 | 4.32 | 4.10 | 0.44 | 4.54 | −0.22 |
| IV | Mar. 12 | | 4.32 | 3.64 | | | |
| | " 13 | | 4.32 | 3.42 | | | |
| | " 14 | | 4.32 | 4.27 | | | |
| Average..... | | 54.0 | 4.32 | 3.78 | 0.44 | 4.22 | +0.10 |
| V | Mar. 15 | | 4.32 | 4.00 | | | |
| | " 16 | | 4.32 | 2.76 | | | |
| | " 17 | | 4.32 | 4.20 | | | |
| Average..... | | 54.0 | 4.32 | 3.65 | 0.35 | 4.00 | +0.32 |
| Average of all periods..... | | 54.0 | 4.32 | 3.87 | 0.39 | 4.26 | +0.06 |
| Daily intake and output of nitrogen in Experiment II. Subject A., milk diet. | | | | | | | |
| I | Mar. 18 | | 4.15 | 3.79 | | | |
| | " 19 | | 4.15 | 3.06 | | | |
| | " 20 | | 4.15 | 3.25 | | | |
| Average..... | | 54.0 | 4.15 | 3.37 | 0.32 | 3.69 | +0.46 |

TABLE VIII—*Continued.*

| Period. | Date. | Body weight. | Nitrogen. | | | | |
|---|---|---|---|---|---|---|---|
| | | | In food. | In urine. | In feces. | Output. | Balance. |
| **Experiment II—Continued.** | | | | | | | |
| | | *kg.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* |
| II | Mar. 21 | | 4.15 | 3.78 | | | |
| | " 22 | | 4.15 | 3.68 | | | |
| | " 23 | | 4.15 | 3.38 | | | |
| Average..... | | 54.0 | 4.15 | 3.61 | 0.44 | 4.05 | +0.1 |
| III | Mar. 24 | | 4.15 | 3.06 | | | |
| | " 25 | | 4.15 | 2.66 | | | |
| | " 26 | | 4.15 | 3.03 | | | |
| Average..... | | 54.0 | 4.15 | 2.92 | 0.33 | 3.25 | +0.9 |
| IV | Mar. 27 | | 4.15 | 3.22 | | | |
| | " 28 | | 4.15 | 2.91 | | | |
| | " 29 | | 4.15 | 2.91 | | | |
| Average..... | | 54.0 | 4.15 | 3.01 | 0.39 | 3.40 | +0.75 |
| Average of all periods..... | | 54.0 | 4.15 | 3.23 | 0.37 | 3.60 | +0.55 |
| **Daily intake and output of nitrogen in Experiment III. Subject A., bread and milk diet.** | | | | | | | |
| I | Mar. 30 | | 4.12 | 3.11 | | | |
| | " 31 | | 4.12 | 3.04 | | | |
| | Apr. 1 | | 4.12 | 3.22 | | | |
| Average..... | | 54.0 | 4.12 | 3.12 | 0.39 | 3.51 | +0.61 |
| II | Apr. 2 | | 4.12 | 3.14 | | | |
| | " 3 | | 4.12 | 3.51 | | | |
| | " 4 | | 4.12 | 3.36 | | | |
| Average..... | | 54.0 | 4.12 | 3.34 | 0.38 | 3.72 | +0.4 |
| III | Apr. 5 | | 4.12 | 3.31 | | | |
| | " 6 | | 4.12 | 3.24 | | | |
| | " 7 | | 4.12 | 3.11 | | | |
| Average..... | | 54.0 | 4.12 | 3.22 | 0.65 | 3.87 | +0.25 |
| IV | Apr. 8 | | 4.12 | 3.33 | | | |
| | " 9 | | 4.12 | 3.08 | | | |
| | " 10 | | 4.12 | 3.15 | | | |
| Average..... | | 54.0 | 4.12 | 3.19 | 0.58 | 3.77 | +0.35 |
| Average of all periods..... | | 54.0 | 4.12 | 3.22 | 0.50 | 3.72 | +0.40 |

TABLE VIII—*Continued.*

| Period. | Date. | Body weight. | Nitrogen. | | | | |
|---|---|---|---|---|---|---|---|
| | | | In food. | In urine. | In feces. | Output. | Balance. |
| Daily intake and output of nitrogen in Experiment IV. Subject B., milk diet. | | | | | | | |
| | | *kg.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* |
| I | Mar. 12 | 63.6 | 4.50 | 6.48 | | | |
| | " 13 | 63.4 | 4.50 | 4.81 | | | |
| | " 14 | 63.0 | 4.50 | 4.77 | | | |
| Average..... | | 63.3 | 4.50 | 5.35 | 0.87 | 6.22 | −1.72 |
| II | Mar. 15 | 63.3 | 4.50 | 6.32 | | | |
| | " 16 | 63.0 | 4.50 | 5.62 | | | |
| | " 17 | 63.0 | 4.50 | 5.86 | | | |
| Average..... | | 63.0 | 4.50 | 5.93 | 0.62 | 6.55 | −2.05 |
| III | Mar. 18 | 62.8 | 4.82 | 4.41 | | | |
| | " 19 | 62.8 | 4.82 | 4.47 | | | |
| | " 20 | 62.8 | 4.82 | 4.15 | | | |
| Average..... | | 62.8 | 4.82 | 4.34 | 0.59 | 4.92 | −0.11 |
| IV | Mar. 21 | 62.9 | 4.82 | 4.18 | | | |
| | " 22 | 62.9 | 4.82 | 4.06 | | | |
| | " 23 | 62.7 | 4.82 | 4.04 | | | |
| Average..... | | 62.8 | 4.82 | 4.09 | 0.61 | 4.70 | +0.12 |
| V | Mar. 24 | 62.4 | 4.82 | 4.03 | | | |
| | " 25 | 62.7 | 4.82 | 4.03 | | | |
| | " 26 | 62.7 | 4.82 | 4.02 | | | |
| Average..... | | 62.6 | 4.82 | 4.03 | 0.67 | 4.70 | +0.12 |
| Average of last three periods... | | 62.7 | 4.82 | 4.15 | 0.62 | 4.77 | +0.05 |
| Daily intake and output of nitrogen in Experiment V. Subject B., meat diet. | | | | | | | |
| I | Mar. 27 | 62.5 | 5.06 | 5.47 | | | |
| | " 28 | 62.5 | 5.06 | 4.66 | | | |
| | " 29 | 62.7 | 5.06 | 3.59 | | | |
| Average..... | | 62.6 | 5.06 | 4.57 | 0.70 | 5.27 | −0.21 |

TABLE VIII—*Continued.*

| Period. | Date. | Body weight. | Nitrogen. | | | | |
|---|---|---|---|---|---|---|---|
| | | | In food. | In urine. | In feces. | Output. | Balance. |
| **Experiment V—Continued.** | | | | | | | |
| | | *kg.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* |
| II | Mar. 30 | 62.3 | 5.06 | 4.62 | | | |
| | " 31 | 62.6 | 5.06 | 3.38 | | | |
| | Apr. 1 | 62.7 | 5.06 | 3.61 | | | |
| Average..... | | 62.5 | 5.06 | 3.87 | 0.53 | 4.40 | +0.66 |
| III | Apr. 2 | 63.0 | 5.06 | 3.86 | | | |
| | " 3 | 62.8 | 5.06 | 4.10 | | | |
| | " 4 | 62.5 | 5.06 | 4.18 | | | |
| Average..... | | 62.8 | 5.06 | 4.05 | 0.71 | 4.76 | +0.30 |
| IV | Apr. 5 | 62.4 | 5.06 | 5.76 | | | |
| | " 6 | 62.5 | 5.06 | 4.11 | | | |
| | " 7 | 62.6 | 5.06 | 4.39 | | | |
| Average..... | | 62.5 | 5.06 | 4.75 | 0.73 | 5.48 | −0.42 |
| Average of all three periods.. | | 62.6 | 5.06 | 4.31 | 0.67 | 4.98 | +0.08 |
| **Daily intake and output of nitrogen in Experiment VI. Subject C., milk diet.** | | | | | | | |
| I | Mar. 12 | 70.6 | 4.72 | 4.89 | | | |
| | " 13 | 70.4 | 4.72 | 4.77 | | | |
| | " 14 | 69.3 | 4.72 | 4.76 | | | |
| Average..... | | 70.1 | 4.72 | 4.81 | 0.57 | 5.38 | −0.66 |
| II | Mar. 15 | 69.3 | 4.72 | 4.63 | | | |
| | " 16 | 69.3 | 4.72 | 4.69 | | | |
| | " 17 | 70.3 | 4.72 | 4.04 | | | |
| Average..... | | 69.6 | 4.72 | 4.45 | 0.60 | 5.05 | −0.33 |
| III | Mar. 18 | 70.4 | 5.25 | 4.09 | | | |
| | " 19 | 69.3 | 5.25 | 4.37 | | | |
| | " 20 | 69.2 | 5.25 | 4.12 | | | |
| Average..... | | 69.6 | 5.25 | 4.19 | 0.70 | 4.89 | +0.36 |

TABLE VIII—*Continued.*

| Period. | Date. | Body weight. | Nitrogen. | | | | |
|---|---|---|---|---|---|---|---|
| | | | In food. | In urine. | In feces. | Output. | Balance. |
| Experiment VI—Continued. | | | | | | | |
| | | *kg.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* |
| IV | Mar. 21 | 69.3 | 5.25 | 3.92 | | | |
| | " 22 | 69.6 | 5.25 | 4.02 | | | |
| | " 23 | 69.9 | 5.25 | 4.24 | | | |
| Average..... | | 69.6 | 5.25 | 4.06 | 0.67 | 4.73 | +0.52 |
| V | Mar. 24 | 70.0 | 5.25 | 4.10 | | | |
| | " 25 | 69.5 | 5.25 | 4.27 | | | |
| | " 26 | 69.0 | 5.25 | 4.33 | | | |
| Average..... | | 69.5 | 5.25 | 4.23 | 0.51 | 4.74 | +0.51 |
| Average of last three periods... | | 69.6 | 5.25 | 4.16 | 0.63 | 4.79 | +0.46 |
| Daily intake and output of nitrogen in Experiment VII. Subject C., meat diet. | | | | | | | |
| I | Mar. 27 | 70.0 | 5.54 | 3.60 | | | |
| | " 28 | 70.4 | 5.54 | 4.01 | | | |
| | " 29 | 70.2 | 5.54 | 4.31 | | | |
| Average..... | | 70.3 | 5.54 | 3.97 | 0.85 | 4.82 | +0.72 |
| II | Mar. 30 | | 5.54 | 6.26 | | | |
| | " 31 | 70.4 | 5.54 | 3.65 | | | |
| | Apr. 1 | 70.0 | 5.54 | 5.25 | | | |
| Average..... | | 70.2 | 5.54 | 5.05 | 0.64 | 5.69 | −0.15 |
| III | Apr. 2 | 68.7 | 5.54 | 4.92 | | | |
| | " 3 | 69.6 | 5.54 | 4.32 | | | |
| | " 4 | 69.6 | 5.54 | 5.08 | | | |
| Average..... | | 69.3 | 5.54 | 4.77 | 0.88 | 5.65 | −0.11 |
| IV | Apr. 5 | 68.9 | 5.54 | 4.67 | | | |
| | " 6 | 68.9 | 5.54 | 5.59 | | | |
| | " 7 | 69.6 | 5.54 | 5.39 | | | |
| Average..... | | 69.1 | 5.54 | 5.22 | 0.78 | 6.00 | −0.46 |
| Average of all periods..... | | 69.7 | 5.54 | 4.75 | 0.79 | 5.54 | 0.00 |

TABLE VIII—*Concluded.*

| Period. | Date. | Body weight. | Nitrogen. | | | | |
|---|---|---|---|---|---|---|---|
| | | | In food. | In urine. | In feces. | Output. | Balance. |
| Daily intake and output of nitrogen in Experiment VIII. Subject D., soy bean curd diet. | | | | | | | |
| | | *kg.* | *mg.* | *gm.* | *gm.* | *gm.* | *gm.* |
| I | Mar. 28 | | 4.00 | 3.85 | | | |
| | " 29 | | 4.00 | 3.27 | | | |
| | " 30 | | 4.00 | 3.00 | | | |
| Average..... | | 50.4 | 4.00 | 3.37 | 0.89 | 4.26 | −0.26 |
| II | Mar. 31 | | 4.00 | 3.05 | | | |
| | Apr. 1 | | 4.00 | 3.37 | | | |
| | " 2 | | 4.00 | 2.74 | | | |
| Average..... | | 50.4 | 4.00 | 3.05 | 1.12 | 4.17 | −0.17 |
| III | Apr. 3 | | 4.00 | 2.94 | | | |
| | " 4 | | 4.00 | 2.74 | | | |
| | " 5 | | 4.00 | 2.68 | | | |
| Average..... | | 50.4 | 4.00 | 2.79 | 0.80 | 3.59 | +0.41 |
| IV | Apr. 6 | | 4.00 | 2.46 | | | |
| | " 7 | | 4.00 | 2.63 | | | |
| | " 8 | | 4.00 | 3.29 | | | |
| Average..... | | 50.4 | 4.00 | 2.79 | 1.06 | 3.86 | +0.15 |
| Average of all periods..... | | 50.4 | 4.00 | 3.00 | 0.97 | 3.97 | +0.03 |

for C. On the meat diets, the excess of base was slightly less (equivalent to 15 and 16 cc. of normal acid) and Subject B showed considerably higher total acidity than C. In both cases, in spite of the excess base in the diet the output of ammonia was higher on the meat than on the milk diet, being 21 per cent higher for B and 16 per cent higher for C.

The creatinine output was normal, averaging 21 mg. per kilo for B and 16 for C. The slightly higher values on the meat diet were undoubtedly due to the preformed creatinine of the diet. There was no evidence that the body protein was being catabolized at an unusual rate on either diet.

SUMMARY.

Eight experiments, each of from 12 to 15 days duration, have been conducted on four young women, to test the relative efficiency of milk, meat, bread and milk, and soy bean curd in maintaining nitrogen equilibrium in the adult human subject. The same basal ration was used throughout, with the energy yield adjusted to maintain body weight. The protein intake was in each instance approximately 0.5 gm. per kilo of body weight (range 0.461 to 0.506 gm. of protein or 0.075 to 0.081 gm. of nitrogen) and in no instance was there a negative nitrogen balance, although the intake was from 15 to 20 per cent less than the average for maintenance on a mixed diet as determined by Sherman. Two subjects were on practically the same level of protein intake for 24 days each and one for 39 days. The food under investigation in each case furnished from 97 to 98 per cent of the total protein of the diet.

Changing from meat to milk and then to bread and milk (Subject A) resulted in average daily storage on meat of 0.06 gm. of protein, on milk of 0.55 gm., and on bread and milk of 0.40 gm.; in other words, 1.4 per cent of the total intake was stored on meat, 13.3 per cent on milk, and 9.7 per cent on bread and milk. Changing from milk to meat made no practical difference as compared with changing from meat to milk (Subjects B and C). These two subjects were close to equilibrium throughout: Subject C stored more on milk than on meat in spite of a slightly higher protein intake on the meat diet; Subject B was so close to equilibrium in both cases that the difference is insignificant.

Fecal nitrogen was very uniform throughout the milk, meat, and bread and milk experiments, and very low as compared with the reports of other investigators, being from 11 to 12 per cent of the total intake. The "cost of digestion" was considerably greater on the soy bean diet, the fecal nitrogen being 24 per cent of the intake.

The urinary nitrogen was practically the same for all subjects on the milk, bread and milk, and soy bean diet, averaging 0.06 gm. per kilo; on the meat diet it was higher, averaging 0.07 gm. per kilo.

The results are not complicated by great differences in the acid-

base balance of the diet, as shown by studies of acidity of the urine and output of ammonia. The ammonia in the urine was somewhat higher on the meat than on the milk diet, being in one case 16 per cent and in the other 21 per cent above the level on the milk diet. This does not seem to be accounted for by difference in acidity of the urine. Determinations of creatinine in the urine of these subjects indicate that there was no essential difference in the metabolism of the different periods.

The evidence on the whole indicates that the nitrogen balance can be maintained with milk, meat, or white bread and milk in amounts to contribute each half of the protein, with a protein intake which is not over 85 per cent of the average maintenance requirement of adults, but that the efficiency of meat is slightly less than that of milk or bread and milk. The high fecal nitrogen on the soy bean diet may be interpreted to mean a lower biological value, although the soy bean must be regarded as very efficient, inasmuch as equilibrium was maintained on a very low intake. This point requires further investigation.

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# INSULIN AND PHLORHIZIN DIABETES.

## SECOND PAPER.

## WITH SOME FURTHER OBSERVATIONS ON THE MECHANISM OF PHLORHIZIN DIABETES.

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In the first paper of this series (1) were reported some preliminary observations of the action of insulin in fasting phlorhizinized dogs. Among other results a transitory sparing action upon the protein metabolism was indicated, but this effect could not be attributed to an appreciable combustion of carbohydrate. It seemed more likely that the administration of insulin in these experiments led to a concentration of carbohydrate in the metabolizing tissues, probably in the form of glycogen; or induced an increased or more complete metabolism of fat. Either or both of these effects would account for the phenomena observed.

Ringer (2), in similar experiments carried out at practically the same time, reached the conclusion that insulin injected in completely phlorhizinized dogs results in an oxidation of glucose. Indeed, Ringer claims that insulin completely corrects phlorhizin diabetes in so far as the condition may be taken as an inability to oxidize sugar. On the basis of his results the mechanism of phlorhizin action is suggested as being a localized injury to the pancreas, preventing the production of the antidiabetic hormone.[1]

[1] This theory of phlorhizin action is no longer tenable since Nash and Benedict (3), and Cori (4), have shown that phlorhizin produces no appreciable change in the insulin content of the pancreas. It may be noted, however, that Ets (5) accepts the theory as best explaining his own results upon phlorhizinized animals.

The experiments of Ringer differed from those of the present writer mainly in the details that Ringer gave glucose with insulin to his animals, and in one instance followed the respiratory exchange and heat production in the Lusk calorimeter. His conclusions depend upon a failure to recover considerable amounts of the sugar ingested; an elevation of the respiratory quotient; and, incidentally, such effects as a decrease in the protein catabolism and the abolition of ketonuria.

The accuracy of sugar recoveries in Ringer's experiments has been criticized by Colwell (6), who called attention to the obvious lack of adequate after periods and controls. Colwell, working with phlorhizinized dogs, found very little net effect of insulin. We cannot agree with Colwell, however, that the observed depressions in nitrogen and sugar output are attributable to simple retentions due to injury to the kidneys or to the tissues at large. Not only is it well known that glucose spares the protein metabolism in phlorhizinized animals, but we have found also that the decreases in nitrogen and sugar output which result from insulin injection alone coincide with a lowered concentration of these substances in the blood.

Abolition of ketonuria in phlorhizinized dogs under the influence of insulin can no longer be taken as evidence of carbohydrate combustion. It has been known for some time that simple meat feeding to phlorhizinized dogs abolishes ketosis, although none of the sugar arising from the protein is burned (7). We have found in many experiments during the past 2 years that glucose given to phlorhizinized dogs and quantitatively recovered (within the accepted limitations of the term) likewise abolishes ketosis.[2]

The elevation in respiratory quotient observed by Ringer is the most substantial evidence that insulin caused his phlorhizinized dogs to burn carbohydrate. Before proceeding with a discussion of this point, however, we shall refer briefly to several other

[2] While we were engaged in writing this paper a report by Wierzuchowski (8) appeared announcing similar observations. The earlier findings of Allen and Wishart (9) were suggestive but not conclusive, since the animals employed were apparently not maximally phlorhizinized and no attempt was made to find whether the sugar given was quantitatively eliminated. In fact, the latter authors claim that an established ketonuria may not be immediately abolished by the feeding of protein or carbohydrate.

papers which have since appeared with observations of the effect of insulin in phlorhizin diabetes.

Cori (10), working with phlorhizinized rabbits, found that insulin caused a retention of sugar which could not be accounted for by its combustion. He concluded that the sugar is stored as glycogen or fat; but a sufficient after period was not allowed to determine whether the retained sugar was subsequently excreted.

Page (11) found elevations in the respiratory quotients of phlorhizinized dogs after giving 40 gm. doses of sucrose and 3 units of insulin. The technique of administering phlorhizin, and the glucose-nitrogen ratios in the experiments, indicate that the animals were by no means completely diabetic. This presumption is strengthened by the fact that in two of the dogs 40 gm. doses of sucrose given as late as 48 hours after the very small injections of insulin raised the respiratory quotient to a level quite comparable with that obtained when sucrose and insulin were given together. Page further refers to a hyperpnea which was usually present at the time the elevated respiratory quotients were observed, and might have been responsible for a temporary blowing off of $CO_2$.

Gaebler and Murlin (12), and Gaebler (13), found that insulin elevates the respiratory quotient in phlorhizinized dogs; the sugar which disappears from the urine and does not reappear may amount to from 0.28 to 0.48 gm. per clinical unit of insulin. They found evidence of retention of sugar as well as of its combustion, but were unable to decide whether the effect on combustion was direct or indirect.

It may perhaps be accepted, in view of the evidence cited, that elevation of the respiratory quotient is one of the fixed results of insulin administration to completely phlorhizinized dogs. Quite as well established, however, is the fact that a relative increase in carbohydrate combustion is not the only mechanism which may operate to raise the respiratory quotient in non-phlorhizinized animals. The literature abounds with cautions in the interpretation of the respiratory exchange. Thus, Lusk (14) warns ". . . . against the too literal interpretation of respiratory quotients obtained under grossly abnormal circumstances." Dickson, Eadie, Macleod, and Pember (15) believe that ". . . . much weight should not be placed on . . . . computations . . . . based very largely on the respiratory quotient, alterations in which depend not merely on the relative combustion of carbohydrates but also on intermediary changes concerned in their reduction into substances related to fatty acids." Boothby and Weiss (16), who observed a drop in the $CO_2$-combin-

ing power of the blood of insulinized dogs, resulting in increased $CO_2$ elimination, state: "It is obvious that until further quantitative relationships are established between the $CO_2$-combining power and the rise of the respiratory quotient it is not justifiable to draw deductions from the respiratory quotient in regard to the effect of insulin on the metabolic processes." Briggs, Koechig, Doisy, and Weber (17) claim that insulin causes an increase in lactic acid in the blood of normal animals paralleling the decrease in glucose, with the result that sufficient $CO_2$ is liberated to affect the study of respiratory data on animals following the use of insulin. Palmer (18) has called attention to the fact that:

> "Under the usual conditions there is a possible error of 6 to 10 per cent in the determination of the respiratory quotient because the second figure in the quotient is accurate to within 2 or 3 only, although under ideal circumstances an error of only 1 in the second place may be secured. Hence a man could burn 50 gm. of dextrose at the rate of 2 gm. per hour in 24 hours, impossible of detection by the calorimeter. On the other hand, an organism may be accredited with the combustion of more sugar than actually occurs, at least within these limits. In diabetes the situation is further complicated when acidosis is present."

The two most important mechanisms, other than increased carbohydrate combustion, which may operate to raise the respiratory quotient have now been suggested. One possibility is the formation of fat or some other reduction product of carbohydrate; another possibility is the production of lactic acid or some other acid, with resultant hyperpnea and blowing out of "extra" $CO_2$. The literature on these two points is unusually conflicting; the one definite conviction we get from the abundant insulin experimentation is that the trend of opinion is away from the earlier view that the main influence of insulin upon carbohydrate combustion is direct.

In calorimeter experiments upon phlorhizinized animals, under the conditions employed by Ringer (2), not only do the foregoing general considerations apply, but certain other more limited ones as well. Ringer gave to his dog before the calorimeter periods large doses of glucose and insulin, either or both of which, as we show in the present paper, abolishes the ketosis and depresses the protein catabolism. Either or both of these effects would be

expected to elevate the respiratory quotient to some extent. Thus, the calculated respiratory quotient for acetoacetic acid is 1.0; for beta-hydroxybutyric acid, 0.888; and for acetone, 0.750. Lusk (19) has calculated the value of the diabetic respiratory quotient for protein as 0.64; therefore a relative decrease in the protein fraction of the total metabolism should operate in the direction of elevating the total respiratory quotient. The question may well be raised whether respiratory-calorimetric experiments upon the effect of glucose in phlorhizin diabetes have been comprehensive enough to justify the generally accepted conclusion that the effect is nil. In Ringer's calorimeter experiment, for example, it may be observed that on May 23 the basal non-protein respiratory quotient was approximately 0.75, a value which, on the basis of the calculations employed in the subsequent glucose-insulin periods, indicates the hourly combustion of about 0.7 gm. of carbohydrate. On May 24, when 40 gm. of glucose were given, the non-protein respiratory quotient was, during 2 hours of the calorimeter period, 0.74. In the subsequent periods, when glucose and insulin had been administered, we feel that the distribution in calculation between the protein and non-protein calories and respiratory exchange is open to objection. As indicated in our first paper on this subject (1), and fully confirmed by our findings reported herein, the nitrogen excretion must have been steadily falling during the calorimeter periods. Furthermore, due to the nitrogen lag (Nash and Benedict (20)), the nitrogen output of the relatively short calorimeter period does not reflect accurately the protein catabolism of that period. It should be noted, also, that Ringer has employed in his calculations factors which apply in complete diabetes (Lusk (14)), despite his claim that insulin had corrected, in considerable part at least, the diabetic condition of the animal.

With these various considerations in mind, and with the idea of following out further the possibilities suggested by our earlier work, we have performed during the past 2 years a rather extensive series of experiments upon the action of insulin and glucose, combined and separately, in phlorhizin diabetes of dogs. We have sought:

1. To compare the effects upon the protein metabolism and

ketosis of glucose given alone and with insulin, in the same animal.

2. To compare the recoveries of glucose given alone and with insulin, in the same animal, with adequate after periods.

3. To find whether sugar withdrawn from the blood under the influence of insulin is stored in the tissues as glycogen in amount sufficient to indicate the probability of fat formation.

4. To find whether glucose alone given to phlorhizinized dogs leads to appreciable glycogen storage.

### *General Procedure and Methods.*

The experimental animals were well conditioned female dogs. Merck's phlorhizin was used, and injected subcutaneously in olive oil suspension. In general, where the experimental period was to be prolonged, we sought to shorten the preliminary fasting and phlorhizin periods. In one or two instances the preliminary regimen was apparently not sufficient to reduce the glucose-nitrogen ratios to the proper control level; but altogether our schedule operated very satisfactorily in this respect, and served to sustain the physical condition of the animals in the latter stages of the experiments. In a wide experience with fasting phlorhizinized dogs we have found that the duration of the period in which reliable results may be anticipated is rather sharply limited.

Merck's "highest purity, anhydrous" dextrose was employed. Repeated analyses of this product by the same method as employed for the urine sugar determinations indicated it to be between 99 and 100 per cent pure glucose.

The insulin preparation used was U-10 Iletin, for a generous supply of which we are indebted to Eli Lilly and Company.

Analyses were made in duplicate. Total nitrogen of the urine was determined by the usual macro Kjeldahl method; non-protein nitrogen of the blood in the Folin-Wu filtrate by a micro Kjeldahl-distillation-Nesslerization technique; urine sugar by Allihn's method; blood sugar by the Folin-Wu method; total acetone bodies of the urine and blood, expressed as acetone, by the methods of Van Slyke (21) and Van Slyke and Fitz (22); inorganic phosphate of the urine by uranium acetate titration; blood phosphates by the method of Benedict and Theis (23).

Glycogen content of muscle[3] and liver tissues was determined by Pflü-

[3] There is a very striking and interesting difference in the character of reduction of Allihn's tartrate-copper reagent by the hydrolyzed glycogen of muscle and liver. It may be recalled that in Pflüger's method for the estimation of glycogen, the tissues are first digested on the water bath in 60 per cent KOH, the glycogen is precipitated by alcohol and recovered by filtering (in our procedure by centrifuging). The glycogen is then repeatedly washed with alcohol and ether, and finally dissolved in hot water and

ger's procedure (25) the semipurified glycogen being converted to glucose by acid hydrolysis and analyzed by the Allihn technique. In isolating and washing the glycogen we substituted, to great advantage, centrifuging for filtering. The dogs whose tissues were taken for glycogen estimation were stunned by a blow on the head, and a chloroform mask was then applied. Liver and muscle tissues were removed as rapidly as possible, approximately 100 gm. portions weighed to an accuracy of 0.1 gm., cut into small pieces, and placed in 60 per cent potassium hydroxide solution which had been brought to the temperature of the boiling water bath. The time elapsing between striking the animal and beginning the digestion of the liver sample was in no case more than 2 minutes; removal of the muscle required a slightly longer time, but was usually accomplished within 5 minutes.

## EXPERIMENTAL.

Essential data of several experiments are shown in typical protocols, Tables I to X inclusive, which illustrate, also, the

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hydrolyzed by heating with hydrochloric acid. After neutralizing and filtering, the solution may be analyzed for glucose by the same procedure we have employed upon the urines of the phlorhizinized dogs. After some experience with this Allihn's method one is able to estimate roughly the sugar concentration of the solution analyzed from the rapidity with which reduction begins in the alkaline copper mixture. The glucose solutions obtained from liver glycogen behave in a perfectly normal manner. Solutions prepared in exactly the same way from muscle glycogen, however, reduce the Allihn's solution at a rate many times greater than normally corresponds to the extent of reduction. One would infer in such instances a sugar concentration three to five times the actual concentration. Furthermore, the appearance of the reduced copper compound is entirely different in the two cases. With glucose from liver glycogen the cuprous oxide has the normal red color; but when the solution of glucose from muscle glycogen is run into the boiling Allihn's solution, almost instantaneously a greenish yellow cloud flashes up in the mixture, the color changing quickly to a brownish yellow which is the final color of the precipitate. The phenomenon is somewhat similar to that occasionally observed with concentrated urines high in creatinine. We have repeatedly found, however, that the nitrogen content of the glucose preparations employed was negligible, and we were never able to demonstrate a trace of creatinine by the Jaffe reaction. The phenomenon interested us sufficiently to lead to further work, which so far has served to show only that the interfering substance may be separated from the glycogen before hydrolysis of the latter, and is possibly a lipoid. The accuracy of the Allihn's method for estimating the sugar from muscle glycogen, without removal of the "interfering" substance, we have abundantly confirmed by many independent analyses by various other methods (including a new technique recently reported from this laboratory (24)).

general plan and objectives of the work. The great majority of urine analyses, particularly during the day periods, were made of catheter specimens taken at 2 hour intervals for as long as changes in composition were likely to be observed. The very frequent urine examinations ensured that temporary effects would not be masked by subsequent compensatory readjustments. To conserve space in the protocols, the results of many short urine periods, which did not show significant variations in any of the critical values, have been averaged for longer periods.

TABLE I.

Dog. 12. Female. Weight 8 kilos. Fasted for 48 hrs. before, and during, the experimental period. Daily subcutaneous injections of 1 gm. of phlorhizin in olive oil at 8.10 a.m., beginning Jan. 10.

| Date. | Time of collection. | Urine. | | | | |
|---|---|---|---|---|---|---|
| | | Reaction to litmus. | Acetone.* | Total N per hr. | Total sugar per hr. | D:N |
| *1925* | | | | *gm.* | *gm.* | |
| Jan. 11 | 8.00 a.m. to | | | | | |
| " 12 | 8.00 a.m. | Acid. | Positive. | 0.20 | 0.68 | 3.37 |
| Dog was given by stomach tube at 10.05 a.m., Jan. 12, a solution of 20 gm. of glucose in 150 cc. of warm water. | | | | | | |
| Jan. 12 | 10.00 a.m. | Acid. | Positive. | 0.29 | 0.83 | 2.84 |
| | 4.00 p.m. | Neutral. | Negative. | 0.24 | 1.93 | 7.95 |
| " 13 | 3.35 a.m. | Acid. | Positive. | 0.14 | 0.69 | 4.95 |
| | 8.00 " | " | " | 0.22 | 0.83 | 3.69 |
| Total for 24 hrs..... | | | | 4.65 | 24.93 | 5.36 |
| Dog was given by stomach tube at 10.05 a.m., Jan. 13, a solution of 25 gm. of glucose in 100 cc. of warm water; at 1.00 p.m., 25 gm. of glucose in 150 cc. of warm water. Subcutaneous injections of 10 units each of U-10 Iletin at 10.05 a.m., 11.30 a.m., 1.00 p.m., 2.30 p.m. | | | | | | |
| Jan. 13 | 10.00 a.m. | Acid. | Positive. | 0.25 | 0.89 | 3.63 |
| | 12.00 n. | Slightly acid. | " | 0.21 | 2.16 | 10.29 |
| | 5.00 p.m. | Neutral. | Negative. | 0.15 | 1.39 | 9.42 |

Dog killed at 5.15 p.m., Jan. 13. Blood sugar just before death, 0.076 per cent. Liver glycogen, 3.35 per cent; muscle glycogen, 0.540 per cent.

* Nitroprusside test.

TABLE II.

Dog 13. Female. Weight 12.9 kilos. Fasted for 48 hrs. before, and during, the experimental period. Daily subcutaneous injections of 1 gm. of phlorhizin in olive oil. First injection at 5.30 p.m., Apr. 13; subsequent injections at 7.40 a.m.

| Date. | Time of collection. | Urine. | | | | | | Blood per 100 cc. | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Reaction to litmus. | Inorganic P per hr. | Total acetone per hr. | Total N per hr. | Total sugar per hr. | D:N | Inorganic P. | Sugar. | Non-protein N. |
| *1925* | | | *gm.* | *gm.* | *gm.* | *gm.* | | *mg.* | *mg.* | *mg.* |
| Apr. 15 | 7.30 a.m. to | | | | | | | | | |
| " 16 | 7.30 a.m. | Acid. | 0.030 | 0.038 | 0.38 | 1.31 | 3.41 | | | |

Dog was given by stomach tube at 11.30 a.m., Apr. 16, a solution of 30 gm. of glucose in 150 cc. of warm water.

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Apr. 16 | 11.30 a.m. | Acid. | 0.031 | 0.050 | 0.42 | 1.55 | 3.70 | 5.0 | 64 | 35 |
| | 1.30 p.m. | Neutral. | 0.014 | 0.015 | 0.36 | 5.21 | 14.27 | | | |
| | 2.30 " | | | | | | | 3.8 | 136 | 31 |
| | 5.30 " | " | 0.000 | 0.000 | 0.28 | 2.77 | 10.08 | | | |
| | 7.30 " | Slightly acid. | 0.014 | 0.002 | 0.25 | 1.67 | 6.66 | 4.9 | 83 | 28 |
| " 17 | 7.30 a.m. | Acid. | 0.021 | 0.028 | 0.26 | 1.34 | 5.09 | | | |
| Total for 24 hrs. | | | 0.438 | 0.565 | 7.15 | 47.08 | 6.58 | | | |

Dog was given by stomach tube at 11.30 a.m., Apr. 17, a solution of 30 gm. of glucose in 150 cc. of warm water; at 2.30 p.m., 30 gm. of glucose in 150 cc. of warm water. Subcutaneous injections of 10 units each of U-10 Iletin at 11.30 a.m., 1.00 p.m., 2.30 p.m., 4.00 p.m.

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Apr. 17 | 11.30 a.m. | Acid. | 0.027 | 0.106 | 0.38 | 1.39 | 3.70 | 6.4 | 66 | 31 |
| | 1.30 p.m. | Slightly acid. | 0.012 | 0.050 | 0.34 | 4.58 | 13.48 | | | |
| | 2.30 " | | | | | | | 3.2 | 83 | 25 |
| | 5.30 " | Neutral. | 0.000 | 0.005 | 0.22 | 2.55 | 11.72 | | | |
| | 6.00 " | " | | | 0.14 | 0.46 | 3.43 | 3.8 | 50 | 23 |

Dog killed at 6.05 p.m., Apr. 17. Liver glycogen, 3.39 per cent; muscle glycogen, 0.705 per cent.

Our experiments may be divided roughly into two groups: first, those in which glycogen of the tissues was determined

TABLE III.

Dog 14. Female. Weight 11.6 kilos. Fasted for 48 hrs. before, and during, the experimental period. Subcutaneous injections of 1 gm. of phlorhizin in olive oil at 8.10 a.m. and 5.00 p.m., Jan. 31; daily thereafter at 8.10 a.m.

| Date. | Time of collection. | Urine. | | | | | | Blood sugar per 100 cc. |
|---|---|---|---|---|---|---|---|---|
| | | Reaction to litmus. | Acetone.* | Inorganic P per hr. | Total N per hr. | Total sugar per hr. | D:N | |
| *1925* | | | | *gm.* | *gm.* | *gm.* | | *mg.* |
| Feb. 1 | 8.00 a.m. to | | | | | | | |
| " 2 | 10.00 a.m. | Acid. | Positive. | 0.024 | 0.43 | 1.60 | 3.70 | 70 |
| Dog was given by stomach tube at 10.05 a.m., Feb. 2, a solution of 25 gm. of glucose in 150 cc. of warm water. | | | | | | | | |
| Feb. 2 | 12.00 n. | Slightly acid. | ? | 0.019 | 0.42 | 6.01 | 14.47 | 145 |
| | 2.00 p.m. | Neutral. | Negative. | 0.009 | 0.28 | 4.23 | 14.82 | |
| | 6.00 " | Slightly acid. | " | 0.017 | 0.29 | 1.58 | 5.46 | |
| " 3 | 8.00 a.m. | Acid. | Positive. | 0.023 | 0.34 | 1.37 | 3.98 | |
| | 10.00 " | " | " | 0.031 | 0.31 | 1.45 | 4.66 | 57 |
| Total for 24 hrs | | | | 0.508 | 8.95 | 52.31 | 5.84 | |
| Dog was given by stomach tube at 10.05 a.m., Feb. 3, a solution of 30 gm. of glucose in 150 cc. of warm water; at 1.00 p.m., 30 gm. of glucose in 150 cc. of warm water. Subcutaneous injections of 10 units each of U-10 Iletin at 10.05 a.m., 11.30 a.m., 1.00 p.m., 2.30 p.m. | | | | | | | | |
| Feb. 3 | 12.00 n. | Neutral. | ? | 0.010 | 0.32 | 5.59 | 17.45 | |
| | 12.50 p.m. | | | | | | | 151 |
| | 5.00 " | " | Negative. | 0.005 | 0.18 | 3.56 | 19.56 | 50 |

Dog killed at 5.05 p.m., Feb 3. Liver glycogen, 2.88 per cent; muscle glycogen, 0.560 per cent.

* Nitroprusside test.

(Tables I to VI); and second, those in which recovery of glucose given with insulin was followed (Tables VII to X). The first

TABLE IV.

Dog 16. Female. Weight 13.5 kilos. Fasted for 72 hrs. before, and during, the experimental period. Daily subcutaneous injections of 1 gm. of phlorhizin in olive oil. First injection at 5.15 p.m., May 4; subsequent injections at 7.40 a.m., or 9.40 a.m.

| Date. | Time of collection. | Urine. | | | | | | Blood per 100 cc. | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Reaction to litmus. | Inorganic P per hr. | Total acetone per hr. | Total N per hr. | Total sugar per hr. | D:N | Inorganic P. | Sugar. | Non-protein N. |
| *1925* | | | *gm.* | *gm* | *gm.* | *gm.* | | *mg.* | *mg.* | *mg.* |
| May 6 | 9.30 a.m. to | | | | | | | | | |
| " 7 | 9.30 a.m. | Acid. | 0.024 | 0.025 | 0.37 | 1.13 | 3.02 | 6.7 | 68 | 38 |
| Dog was given by stomach tube at 9.30 a.m., May 7, a solution of 30 gm. of glucose in 150 cc. of warm water; at 12.30 p.m., 30 gm. of glucose in 150 cc. of warm water. | | | | | | | | | | |
| May 7 | 11.30 a.m. | Slightly acid. | 0.016 | 0.014 | 0.40 | 3.66 | 9.15 | | | |
| | 12.30 p.m. | | | | | | | 6.1 | 207 | 34 |
| | 1.30 " | Neutral. | 0.005 | 0.000 | 0.27 | 4.99 | 18.83 | | | |
| | 5.30 " | " | 0.000 | 0.000 | 0.20 | 4.05 | 20.01 | 4.8 | 172 | 28 |
| | 9.30 " | Acid. | 0.009 | 0.000 | 0.16 | 2.17 | 13.34 | | | |
| " 8 | 9.30 a.m. | " | 0.010 | 0.002 | 0.14 | 1.42 | 9.99 | 6.9 | 78 | |
| Total for 24 hrs. | | | 0.191 | 0.052 | 4.50 | 59.26 | 13.17 | | | |
| May 8 | 5.30 p.m. | Acid. | 0.013 | 0.015 | 0.20 | 1.08 | 5.31 | | | |
| " 9 | 7.30 a.m. | " | 0.029 | 0.071 | 0.27 | 1.19 | 4.45 | | | |
| | 9.30 " | " | 0.030 | 0.103 | 0.33 | 1.19 | 3.59 | 8.5 | 66 | 28 |
| Total for 24 hrs. | | | 0.568 | 1.327 | 6.05 | 27.75 | 4.58 | | | |
| Dog was given by stomach tube at 9.30 a.m., May 9, a solution of 30 gm. of glucose in 150 cc. of warm water; at 12.30 p.m., 30 gm. of glucose in 150 cc. of warm water. | | | | | | | | | | |
| May 9 | 11.30 a.m. | Slightly acid. | 0.020 | 0.052 | 0.35 | 4.17 | 12.09 | | | |
| | 1.30 p.m. | Neutral. | 0.005 | 0.000 | 0.25 | 6.27 | 25.08 | | | |
| | 3.30 " | " | 0.000 | 0.000 | 0.19 | 5.49 | 28.90 | 5.1 | 233 | 30 |

Dog killed at 3.35 p.m., May 9. Liver glycogen, 1.96 per cent; muscle glycogen, 0.467 per cent.

TABLE V.

**Dog 20. Female. Weight 14.7 kilos. Fasted for 108 hrs. before, and during, the experimental period. Daily subcutaneous injections of 1 gm. of phlorhizin in olive oil. First injection at 5.10 p.m., April 20; subsequent injections at 7.40 a.m.**

| Date. | Time of collection. | Urine. | | | | | | Blood per 100 cc. | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Reaction to litmus. | Inorganic P per hr. | Total acetone per hr. | Total N per hr. | Total sugar per hr. | D:N | Inorganic P. | Sugar. | Non-protein N. |
| *1925* | | | *gm.* | *gm.* | *gm.* | *gm.* | | *mg.* | *mg.* | *mg.* |
| Apr. 22 | 7.30 a.m. to | | | | | | | | | |
| " 23 | 7.30 a.m. | Acid. | 0.029 | 0.092 | 0.42 | 1.54 | 3.64 | | | |
| Dog was given by stomach tube at 11.30 a.m., Apr. 23, a solution of 30 gm. of glucose in 150 cc. of warm water. | | | | | | | | | | |
| Apr. 23 | 11.30 a.m. | Acid. | 0.027 | 0.097 | 0.40 | 1.60 | 4.00 | 7.0 | 62 | 31 |
| | 1.30 p.m. | " | 0.018 | 0.049 | 0.36 | 5.17 | 14.18 | | | |
| | 2.30 " | | | | | | | 7.3 | 207 | 29 |
| | 5.30 " | Neutral. | 0.006 | 0.000 | 0.24 | 3.95 | 16.63 | | | |
| | 7.30 " | Slightly acid. | 0.027 | 0.007 | 0.22 | 1.51 | 6.73 | 6.5 | 76 | 26 |
| " 24 | 7.30 a.m. | Acid. | 0.023 | 0.078 | 0.33 | 1.57 | 4.76 | | | |
| Total for 24 hrs. | | | 0.501 | 1.431 | 7.70 | 54.48 | 7.07 | | | |
| Dog was given by stomach tube at 11.30 a.m., Apr. 24, a solution of 30 gm. of glucose in 150 cc. of warm water; at 2.30 p.m., 30 gm. of glucose in 150 cc. of warm water. | | | | | | | | | | |
| Apr. 24 | 11.30 a.m. | Acid. | 0.023 | 0.174 | 0.40 | 1.39 | 3.52 | 8.2 | 58 | 27 |
| | 2.30 p.m. | | | | | | | 9.7 | 179 | 25 |
| | 3.30 " | Slightly acid. | 0.016 | 0.060 | 0.28 | 5.56 | 19.69 | | | |
| | 6.00 " | Neutral. | 0.000 | 0.000 | 0.19 | 5.46 | 29.04 | 6.2 | 179 | 21 |

Dog killed at 6.10 p.m., Apr. 24. Liver glycogen, 3.65 per cent; muscle glycogen, 0.870 per cent.

group may be subdivided into experiments in which glycogen formation was observed following administration of glucose and insulin (Tables I to III); and experiments in which glycogen formation was determined after glucose alone had been given

TABLE VI.

Dog 21. Female. Weight 14.1 kilos. Fasted for 72 hrs. before, and during, the experimental period. Daily subcutaneous injections of 1 gm. of phlorhizin in olive oil. First injection at 5.30 p.m., May 18; subsequent injections at 7.40 a.m.

| Date. | Time of collection. | Urine. | | | | | | Blood per 100 cc. | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Reaction to litmus. | Inorganic P per hr. | Total acetone per hr. | Total N per hr. | Total sugar per hr. | D:N | Inorganic P. | Sugar. | Non-protein N. |
| *1925* | | | *gm.* | *gm.* | *gm.* | *gm.* | | *mg.* | *mg.* | *mg.* |
| May 20 | 9.30 a.m. to | | | | | | | | | |
| " 21 | 9.30 a.m. | Acid. | 0.039 | 0.139 | 0.46 | 1.65 | 3.62 | 8.3 | 63 | 35 |
| Dog was given by stomach tube at 9.35 a.m., May 21, a solution of 30 gm. of glucose in 150 cc. of warm water; at 12.30 p.m., 30 gm. of glucose in 150 cc. of warm water. | | | | | | | | | | |
| May 21 | 11.30 a.m. | Slightly acid. | 0.033 | 0.074 | 0.43 | 5.22 | 12.20 | | | |
| | 12.20 p.m. | | | | | | | | 174 | 35 |
| | 5.30 " | Neutral. | 0.000 | 0.000 | 0.29 | 6.90 | 24.21 | 5.7 | 141 | 31 |
| | 9.30 " | Slightly acid. | 0.0005 | 0.003 | 0.19 | 1.57 | 8.25 | | | |
| " 22 | 9.30 a.m. | Acid. | 0.018 | 0.066 | 0.26 | 1.41 | 5.46 | 8.3 | 62 | 28 |
| Total for 24 hrs..... | | | 0.284 | 0.958 | 6.44 | 75.07 | 11.65 | | | |
| May 22 | 1.30 p.m. | Acid. | 0.024 | 0.173 | 0.33 | 1.47 | 4.39 | | | |
| " 23 | 9.30 a.m. | " | 0.026 | 0.223 | 0.39 | 1.36 | 3.52 | 7.8 | 68 | 29 |
| Total for 24 hrs..... | | | 0.624 | 5.159 | 9.06 | 33.06 | 3.65 | | | |
| Dog was given by stomach tube at 9.35 a.m., May 23, a solution of 30 gm. of glucose in 150 cc. of warm water; at 12.30 p.m., 30 gm. of glucose in 150 cc. of warm water. | | | | | | | | | | |
| May 23 | 11.30 a.m. | Slightly acid. | 0.012 | 0.103 | 0.36 | 5.17 | 14.36 | | | |
| | 12.25 p.m. | | | | | | | 4.7 | 214 | 30 |
| | 1.30 p.m. | Neutral. | 0.000 | 0.007 | 0.28 | 6.43 | 22.56 | | | |
| | 4.00 " | " | 0.000 | 0.000 | 0.26 | 6.97 | 26.39 | 3.4 | 234 | 28 |

Dog killed at 4.05 p.m., May 23. Liver glycogen, 1.11 per cent; muscle glycogen, 0.497 per cent.

TABLE VII.

Dog 8. Female. Weight 10.8 kilos. Fasted for 36 hrs. before, and during, the experimental period. Daily subcutaneous injections of 1 gm. of phlorhizin in olive oil. First injection at 5.15 p.m., Apr. 12; subsequent injections at 7.10 a.m.

| Date. | Time of collection. | Urine. | | | | | Blood per 100 cc. | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Reaction to litmus. | Total acetone per hr. | Total N per hr. | Total sugar per hr. | D:N | $CO_2$-combining power. | Total acetone. | Sugar. |
| *1924* | | | *gm.* | *gm.* | *gm.* | | *cc.* | *mg.* | *mg.* |
| Apr. 14 | 7.00 a.m. to | | | | | | | | |
| " 15 | 7.00 a.m. | Acid. | 0.080 | 0.39 | 1.38 | 3.53 | | | |

At 11.05 a.m., Apr. 15, injected subcutaneously 20 units of U-10 Iletin. Immediately thereafter the dog drank a solution of 50 gm. of glucose in 200 cc. of warm water. At 1.35 p.m. the dog vomited a volume of 180 cc., containing 3.94 gm. of glucose and 0.03 gm. of nitrogen.

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Apr. 15 | 11.00 a.m. | Acid. | 0.130 | 0.39 | 1.39 | 3.55 | 28 | 25 | 68 |
| | 1.00 p.m. | Slightly acid. | 0.046 | 0.35 | 4.11 | 11.59 | | | |
| | 3.00 " | Neutral. | 0.000 | 0.23 | 4.03 | 17.52 | 48 | 0 | 146 |
| | 9.00 " | " | 0.000 | 0.17 | 1.83 | 10.85 | | | |
| " 16 | 7.00 a.m. | Acid. | 0.003 | 0.14 | 1.28 | 8.88 | | | |
| Total for 24 hrs. | | | 0.639 | 5.19 | 45.62 | 8.79 | | | |
| Apr. 16 | 11.00 a.m. | Acid. | 0.027 | 0.20 | 1.36 | 6.66 | | | |
| " 17 | 7.00 " | " | 0.187 | 0.28 | 1.14 | 4.09 | | | |
| Total for 24 hrs. | | | 3.842 | 6.37 | 28.18 | 4.42 | | | |
| Apr. 17 | 11.00 a.m. | Acid. | 0.243 | 0.32 | 1.14 | 3.60 | | | |
| " 18 | 7.00 " | " | 0.191 | 0.32 | 1.06 | 3.34 | 16 | 38 | 60 |
| Total for 24 hrs. | | | 4.800 | 7.60 | 25.70 | 3.38 | | | |

Experiment discontinued.

(Tables IV to VI). In every animal except Dog 8 (Table VII) the recovery of a preliminary dose of glucose given without insulin, and its effects upon nitrogen elimination, phosphate excretion, urine reaction, and ketonuria, were tested.

TABLE VIII.

Dog. 9. Female. Weight 11.3 kilos. Fasted for 48 hrs. before, and during, the experimental period. Daily subcutaneous injections of 1 gm. of phlorhizin in olive oil. First injection at 5.10 p.m., May 9; subsequent injections at 7.10 a.m.

| Date. | Time of collection. | Urine. | | | | | Blood per 100 cc. | |
|---|---|---|---|---|---|---|---|---|
| | | Reaction to litmus. | Total acetone per hr. | Total N per hr. | Total sugar per hr. | D:N | $CO_2$-combining power. | Sugar. |
| *1924* | | | *gm.* | *gm.* | *gm.* | | *cc.* | *mg.* |
| May 11 | 7.00 a.m. to | | | | | | | |
| " 12 | 7.00 a.m. | Acid. | 0.049 | 0.34 | 1.14 | 3.40 | | |
| Dog was given by stomach tube at 11.10 a.m., May 12, a solution of 40 gm. of glucose in 200 cc. of warm water. | | | | | | | | |
| May 12 | 11.00 a.m. | Acid. | 0.037 | 0.31 | 1.46 | 4.74 | 52 | 77 |
| | 3.00 p.m. | Neutral. | 0.004 | 0.27 | 5.21 | 19.29 | 62 | 182 |
| | 7.00 " | Slightly acid. | 0.000 | 0.22 | 2.12 | 9.52 | | |
| | 11.00 " | Acid. | 0.001 | 0.19 | 1.70 | 8.95 | | |
| " 13 | 7.00 a.m. | " | 0.020 | 0.20 | 1.15 | 5.86 | | |
| Total for 24 hrs. | | | 0.327 | 5.54 | 51.20 | 9.24 | | |
| May 13 | 11.00 a.m. | Acid. | 0.068 | 0.22 | 1.02 | 4.66 | | |
| | 7.00 p.m. | " | 0.109 | 0.20 | 0.93 | 4.60 | | |
| " 14 | 7.00 a.m. | " | 0.193 | 0.24 | 0.91 | 3.74 | | |
| Total for 24 hrs. | | | 3.459 | 5.42 | 22.47 | 4.14 | | |
| Dog was given by stomach tube at 11.05 a.m., May 14, a solution of 40 gm. of glucose in 200 cc. of warm water; 20 units of U-10 Iletin injected subcutaneously. | | | | | | | | |
| May 14 | 11.00 a.m. | Acid. | 0.161 | 0.29 | 0.94 | 3.28 | 38 | 66 |
| | 3.00 p.m. | Neutral. | 0.037 | 0.24 | 2.73 | 11.48 | 57 | 120 |
| | 6.00 " | " | 0.000 | 0.19 | 1.87 | 10.03 | | |
| | 9.45 " | Acid. | 0.000 | 0.13 | 1.18 | 9.23 | | |
| " 15 | 7.00 a.m. | " | 0.003 | 0.17 | 1.14 | 6.60 | | |
| Total for 24 hrs. | | | 0.822 | 4.74 | 35.29 | 7.44 | | |
| May 15 | 11.00 a.m. | Acid. | 0.006 | 0.22 | 1.01 | 4.48 | | |
| | 3.00 p.m. | " | 0.036 | 0.23 | 0.75 | 3.20 | | |
| " 16 | 7.00 a.m. | " | 0.123 | 0.25 | 0.80 | 3.17 | | |
| Total for 24 hrs. | | | 2.130 | 5.90 | 19.81 | 3.36 | | |

Experiment continued during the next 24 hrs. with no further recovery of ingested sugar.

TABLE IX.

Dog 17. Female. Weight 16.5 kilos. Fasted for 36 hrs. before, and during, the experimental period. Daily subcutaneous injections of 1 gm. of phlorhizin in olive oil. First injection at 5.10 p.m., Feb. 27; subsequent injections at 9.10 a.m.

| Date. | Time of collection. | Urine. | | | | | | Blood per 100 cc. | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Reaction to litmus. | Inorganic P per hr. | Total acetone per hr. | Total N per hr. | Total sugar per hr. | D:N | Inorganic P. | Sugar. | Non-protein N. |
| *1925* | | | *gm.* | *gm.* | *gm.* | *gm.* | | *mg.* | *mg.* | *mg.* |
| Mar. 1 | 9.00 a.m. to | | | | | | | | | |
| " 2 | 9.00 a.m. | Acid. | 0.032 | 0.230 | 0.44 | 2.19 | 5.00 | | | |
| Dog was given by stomach tube at 11.05 a.m., Mar. 2, a solution of 30 gm. of glucose in 150 cc. of warm water. | | | | | | | | | | |
| Mar. 2 | 11.00 a.m. | Acid. | 0.023 | 0.330 | 0.50 | 3.02 | 5.97 | 6.5 | 79 | 34 |
| | 2.00 p.m. | | | | | | | 4.8 | 201 | 33 |
| | 3.00 " | Neutral. | 0.005 | 0.132 | 0.41 | 8.18 | 19.95 | | | |
| | 5.00 " | " | 0.004 | 0.032 | 0.30 | 3.94 | 13.13 | | | |
| | 7.00 " | Slightly acid. | 0.024 | 0.036 | 0.32 | 2.24 | 7.11 | 6.8 | 73 | 27 |
| | 11.00 " | Acid. | 0.035 | 0.104 | 0.32 | 2.06 | 6.39 | | | |
| " 3 | 9.00 a.m. | " | 0.057 | 0.546 | 0.57 | 2.47 | 4.35 | | | |
| Total for 24 hrs..... | | | 0.832 | 7.200 | 10.85 | 84.09 | 7.75 | | | |
| Dog was given by stomach tube at 11.05 a.m., Mar. 3, a solution of 30 gm. of glucose in 150 cc. of warm water; at 2.05 p.m., 30 gm. of glucose in 150 cc. of warm water. Subcutaneous injections of 15 units of U-10 Iletin at 11.00 a.m., and 10 units each at 12.30 p.m., 2.00 p.m., 3.30 p.m. | | | | | | | | | | |
| Mar. 3 | 11.00 a.m. | Acid. | 0.045 | 0.518 | 0.69 | 2.58 | 3.71 | 6.9 | 68 | 37 |
| | 1.00 p.m. | Slightly acid. | 0.019 | 0.238 | 0.50 | 4.95 | 9.80 | | | |
| | 1.45 " | | | | | | | 4.2 | 122 | 34 |
| | 5.00 " | Neutral. | 0.000 | 0.040 | 0.36 | 4.85 | 13.65 | | | |
| | 7.00 " | " | 0.000 | 0.013 | 0.25 | 1.99 | 8.14 | 4.2 | 50 | 24 |
| | 11.00 " | Slightly acid. | 0.015 | 0.086 | 0.30 | 2.08 | 6.93 | | | |
| " 4 | 9.00 a.m. | Acid. | 0.039 | 0.282 | 0.52 | 3.00 | 5.80 | | | |
| Total for 24 hrs..... | | | 0.578 | 4.864 | 10.68 | 76.77 | 7.19 | | | |

TABLE IX —*Concluded.*

| Date. | Time of collection. | Urine. | | | | | | Blood per 100 cc. | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Reaction to litmus. | Inorganic P per hr. | Total acetone per hr. | Total N per hr. | Total sugar per hr. | D:N | Inorganic P. | Sugar. | Non-protein N. |
| *1925* | | | *gm.* | *gm.* | *gm.* | *gm.* | | *mg.* | *mg.* | *mg.* |
| Mar. 4 | 11.15 a.m. | | | | | | | 6.3 | 71 | 38 |
| | 1.00 p.m. | Acid. | 0.027 | 0.381 | 0.56 | 2.46 | 4.43 | | | |
| " 5 | 9.00 a.m. | " | 0.046 | 0.434 | 0.63 | 2.24 | 3.54 | | | |
| Total for 24 hrs..... | | | 1.026 | 10.204 | 14.87 | 54.67 | 3.68 | | | |

Experiment discontinued. Animal recovered. An unusual and noteworthy incident of the experiment was the very large volume of urine secreted. Thus, during the last 3 days the volumes were, respectively: 3615 cc., 4920 cc., 4295 cc. The daily volume remained well above 4000 cc. during the succeeding 3 days when the dog was being fed.

Thirteen dogs were thus studied in great detail. Two of these, Dogs 13 and 16, were twice used, an interval for convalescence of 3 months being allowed between the first and second experiments upon each. In the first instance it was possible to follow through sufficient after periods the total elimination of glucose administered with and without insulin; in the second experiments, with comparable preliminary conditions, the two dogs were killed for glycogen determination. The results of the first experiments on Dogs 13 and 16 thus serve as controls for the later experiments upon the same animals.

While the various results of all of our experiments bear upon each other intimately, it seems desirable to discuss them under the following headings:

### *1. Recovery of Ingested Glucose.*

The calculation of "extra" sugar in phlorhizin experiments is probably too well understood to require explanation here. It may be pointed out, however, that such a calculation involves assumptions which are only approximately reliable, and in no case can the calculation be absolute. It is for this reason that recoveries of about 85–105 per cent of the theoretical are generally accepted

TABLE X.

Dog 19. Female. Weight 19.7 kilos. Fasted for 48 hrs. before, and during, the experimental period. Daily subcutaneous injections of 1 gm. of phlorhizin in olive oil. First injection at 5.10 p.m., Mar. 23; subsequent injections at 7.40 a.m.

| Date. | Time of collection | Urine. | | | | | | Blood per 100 cc. | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Reaction to litmus. | Inorganic P per hr. | Total acetone per hr. | Total N per hr. | Total sugar per hr. | D:N | Inorganic P. | Sugar. | Non-protein N. |
| *1925* | | | *gm.* | *gm.* | *gm.* | *gm.* | | *mg.* | *mg.* | *mg.* |
| Mar. 25 | 7.30 a.m. to | | | | | | | | | |
| " 26 | 7.30 a.m. | Acid. | 0.036 | 0.050 | 0.54 | 1.80 | 3.31 | | | |

Dog was given by stomach tube at 11.30 a.m., Mar. 26, a solution of 30 gm. of glucose in 150 cc. of warm water.

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Mar. 26 | 11.30 a.m. | Acid. | 0.035 | 0.078 | 0.56 | 1.89 | 3.36 | 6.8 | 71 | 29 |
| | 1.30 p.m. | Slightly acid. | 0.017 | 0.034 | 0.48 | 6.19 | 12.90 | | | |
| | 2.30 " | | | | | | | 5.8 | 195 | 22 |
| | 3.30 " | Slightly acid. | 0.000 | 0.000 | 0.39 | 7.03 | 18.02 | | | |
| | 5.30 " | Neutral. | 0.005 | 0.000 | 0.34 | 2.44 | 7.18 | | | |
| | 7.30 " | Acid. | 0.016 | 0.003 | 0.35 | 1.78 | 5.01 | 6.9 | 76 | 29 |
| | 11.15 " | " | 0.014 | 0.011 | 0.24 | 1.22 | 5.14 | | | |
| " 27 | 7.30 a.m. | " | 0.030 | 0.104 | 0.46 | 1.70 | 3.74 | | | |
| Total for 24 hrs..... | | | 0.516 | 1.283 | 10.02 | 61.06 | 6.09 | | | |

Dog was given by stomach tube at 11.30 a.m., Mar. 27, a solution of 30 gm. of glucose in 150 cc. of warm water; at 2.30 p.m., 30 gm. of glucose in 150 cc. of warm water. Subcutaneous injections of 15 units of U-10 Iletin at 11.30 a.m., and 10 units each at 1.00 p.m., 2.30 p.m., 4.00 p.m.

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Mar. 27 | 11.30 a.m. | Acid. | 0.038 | 0.123 | 0.44 | 1.67 | 3.80 | 7.3 | 67 | 29 |
| | 1.30 p.m. | Slightly acid. | 0.010 | 0.053 | 0.38 | 4.99 | 12.62 | | | |
| | 2.20 " | | | | | | | 5.1 | 147 | 26 |
| | 5.30 " | Neutral. | 0.000 | 0.000 | 0.26 | 5.17 | 20.27 | | | |
| | 7.00 " | | | | | | | 3.7 | 54 | 21 |
| | 7.30 " | " | 0.000 | 0.002 | 0.17 | 1.25 | 7.55 | | | |
| " 28 | 7.30 a.m. | Acid. | 0.021 | 0.006 | 0.21 | 1.74 | 8.31 | | | |
| Total for 24 hrs..... | | | 0.423 | 0.678 | 6.42 | 60.78 | 9.47 | | | |
| Mar. 28 | 12.07 p.m. | Acid. | 0.007 | 0.006 | 0.22 | 1.65 | 7.48 | 7.1 | 68 | 28 |
| | 8.40 " | " | 0.019 | 0.043 | 0.32 | 1.67 | 5.17 | | | |
| " 29 | 7.30 a.m. | " | 0.046 | 0.136 | 0.43 | 1.41 | 3.27 | | | |
| Total for 24 hrs..... | | | 0.694 | 1.865 | 8.46 | 37.23 | 4.40 | | | |

Experiment discontinued. Animal recovered.

as "quantitative." The difference is so prevailingly on the negative side, however, that we are now inclined to question whether the average discrepancy does not reflect a small utilization (not necessarily oxidation) of the carbohydrate. In any event some discretion, and a review of the entire experiment, should direct the calculation of extra sugar.

The recoveries of glucose given in our experiments are summarized in Table XI. When glucose was given without insulin the extra glucose eliminations were, for the most part, typical of the most successful experiments of this character. On a dynamogenetic basis the discrepancies betwen the amounts of glucose fed and the amounts recovered would seem to be, certainly in the majority of the animals, too small to account for the effects produced upon fat and protein metabolism. In Dogs 13 (Table II) and 14 the percentage recovery of glucose cannot be con-

TABLE XI.

*Recovery of Ingested Glucose in Phlorhizin Diabetes.*

Glucose was given in one dose, or in two doses 3 hrs. apart. Insulin was given in one dose, or in four doses at 90 minute intervals.

| Dog. | Without insulin. | | With insulin. | | | |
|---|---|---|---|---|---|---|
| | Glucose given. | Recovered glucose. | Insulin given. | Glucose given. | Recovered glucose. | Glucose retained per unit. |
| | *gm.* | *per cent* | *units* | *gm.* | *per cent* | *gm.* |
| 12 | 20 | 46.3 | | | | |
| 13 | 30 | 75.7 | | | | |
| 14 | 25 | 76.8 | | | | |
| 16 | 30, 30 | 91.9 | | | | |
| 18* | 25 | 107.3 | | | | |
| 20 | 30 | 88.2 | | | | |
| 21 | 30, 30 | 88.0 | | | | |
| 8 | | | 20 | 46 | 73.6 | 0.61 |
| 9 | 40 | 91.0 | 20 | 40 | 56.0 | 0.88 |
| 13* | 30 | 94.2 | 10, 10, 10, 10 | 30, 30 | 82.9 | 0.25 |
| 16* | 30 | 99.2 | 15, 10, 10, 10, | 30, 30 | 75.0† | 0.33 |
| 17 | 30 | 99.5 | 15, 10, 10, 10 | 30, 30 | 61.0 | 0.52 |
| 19 | 30 | 92.9 | 15, 10, 10, 10 | 30, 30 | 81.5 | 0.25 |

* Protocols not given.

† Includes an estimated output for a short period in which the urine was lost.

sidered as quantitative; such results, however, are not uncommon in dogs which are apparently completely phlorhizinized. The result in Dog 12 we are entirely unable to explain.

When glucose and insulin were given together the amounts of glucose recovered were definitely less. It will be noted, however, that the average value of glucose retained per unit of insulin is considerably below that found by Ringer (2), and agrees more closely with the results of Colwell (6). This is true despite the fact that we have made no correction in our values for the average retention of glucose when insulin is not given. The explanation is undoubtedly that in our experiments we allowed sufficient after periods for the excretion of sugar temporarily stored and held as glycogen. In the majority of our dogs extra sugar elimination continued for 36 to 48 hours after giving the glucose and insulin. This observation is in accord with the preliminary period required to deplete the glycogen reserves and establish stable ratios at the beginning of a phlorhizin experiment.

It is, we think, significant that the largest retentions of sugar were in those dogs which received the glucose and insulin in one dose. Although Allen (26) predicted "with perfect positiveness that insulin will have no influence upon phlorizin glycosuria," we should have been very much surprised if insulin had been found to have no effect whatever in phlorhizin diabetes. Any factor so potent as insulin in the mechanism of carbohydrate metabolism would be expected to have some influence on the handling of carbohydrate material by the organism under any and all conditions, if given in sufficient amount. Although phlorhizin and insulin are obviously antagonistic to each other, this antagonism may not be direct but may operate by blocking some stage either before or after insulin comes into play in the normal process. For example, insulin may favor linkage of sugar to the cell, while phlorhizin blocks such linkage. If insulin fails to lessen appreciably the phlorhizin glycuresis in fasting dogs (Nash (1)) it would seem to us that the view must be taken that phlorhizin and insulin are not opposite in action at the same stage of the carbohydrate process. The failure of insulin in the fasting animal would indicate that *glucose* is necessary in excess to oppose the phlorhizin at some stage, and that then an excess of insulin may force some sugar utilization.

*2. Comparison of the Effects upon the Protein Metabolism of Glucose Given Alone and with Insulin.*

In so far as the effect upon the protein metabolism may be inferred from the nitrogen excretion, we have found no significant difference in the degree of effect of corresponding amounts of glucose when given alone and with insulin. Several illustrations from the protocols suffice on this point. Dog 21 received two 30 gm. doses of glucose at the usual 3 hour interval. The average per hour excretion of nitrogen in the control 24 hour period was 0.46 gm. In one 4 hour period following the glucose ingestion the average nitrogen excretion per hour was 0.19 gm., a decrease of 60 per cent. Dog 16, receiving 60 gm. of glucose in divided doses, showed in the subsequent 12 hour night period an average per hour excretion of 0.14 gm. of nitrogen, as compared with an hourly rate of 0.37 gm. during the preceding 24 hour control period, or a decrease of 62 per cent. In an earlier experiment on Dog 16, the protocol of which is not shown, the same amount of glucose was given with 45 units of insulin, in divided doses, and the maximal depression in nitrogen excretion was to 0.17 gm. per hour from a control rate of about 0.40 gm., a decrease of 58 per cent. The same dog on the preceding day had received a single dose of 30 gm. of glucose, which reduced the nitrogen excretion maximally from 0.52 gm. per hour to 0.29 gm., a decrease of 44 per cent. Dogs 13 (an earlier experiment; protocol not given), 17, and 19, which received 60 gm. doses of glucose and 40 to 45 units of insulin, showed maximal depressions in the nitrogen output of 62, 65, and 62 per cent, respectively. Single doses of 30 gm. of glucose to these dogs on the preceding days had effected maximal reductions in the nitrogen excretion of 35, 33, and 56 per cent, respectively.

A depression in the non-protein nitrogen of the blood coincides with the lowered excretion of nitrogen in the urine.

*3. The Effect upon the Urinary Creatinine of Glucose Given Alone and with Insulin.*

While the estimation of creatinine in diabetic urine is attended by various uncertainties (Blau (27)), we felt that in most instances the concentration of interfering substances would be too small to affect seriously the accuracy of the determination. We desired

not only to find whether there was any specific effect of our procedure upon creatinine metabolism, but particularly to confirm whether there was any indication of kidney injury resulting in a general retention of metabolic end-products. We therefore made creatinine estimations on the several urine samples from a number of the dogs in our experiments, and several of these results are assembled in Table XII. The values for each dog are given in the same successive order of urine periods as shown in the general protocols. With the exception of a few pronounced irregularities,

TABLE XII.

*Creatinine Excretion (Expressed in Gm. per Hr).*

| Dog 13, Table II. | Dog 16, Table IV. | Dog 19, Table X. | Dog 20, Table V. | Dog 21, Table VI. |
|---|---|---|---|---|
| 0.017 | 0.016 | 0.024 | 0.017 | 0.017 |
| 0.016 | 0.020 | 0.023 | 0.017 | 0.019 |
| 0.017 | 0.016 | 0.028 | 0.022 | 0.022 |
| 0.015 | 0.018 | 0.026 | 0.018 | 0.017 |
| 0.015 | 0.017 | 0.020 | 0.017 | 0.016 |
| 0.015 | 0.017 | 0.022 | 0.017 | 0.018 |
| 0.017 | 0.018 | 0.015 | 0.017 | 0.016 |
| 0.020 | 0.014 | 0.011* | 0.026 | 0.022 |
| 0.015 | 0.015 | 0.036† | 0.016 | 0.020 |
| — | 0.021 | 0.027 | | 0.026 |
| | 0.022 | 0.025 | | |
| | 0.021 | 0.020 | | |
| | | 0.021 | | |
| | | 0.018 | | |
| | | 0.019 | | |
| | | 0.019 | | |

* Urine of night period.

† Urine of early morning period.

which may be attributable to the interfering effect of sugar or ketone bodies, we believe the results indicate that there was no significant effect upon the creatinine metabolism, and support our conclusion that there was no injury to the kidneys in our animals.

*4. Effect upon Phosphate Excretion of Glucose Given Alone and with Insulin.*

A striking effect of glucose ingestion by the phlorhizinized dogs of our experiments was the sharp reduction in, or complete aboli-

tion of, phosphate excretion in the urine. The phenomenon very closely parallels the urine reaction, which changes from strongly acid to very slightly acid, or more frequently becomes and remains neutral, during the period of heavy sugar excretion. The larger the dosage of sugar the more pronounced and prolonged is the effect upon the urine phosphates. Phosphate elimination recovers usually before that of nitrogen, but there is little indication of an increased compensatory phosphate excretion such as noted by Allan, Dickson, and Markowitz (28) and Eadie, Macleod, and Noble (29). When insulin and glucose are given the effect upon the phosphate excretion is usually more prolonged.

The fall in urine phosphate is associated with an increased $CO_2$-combining power of the blood, and usually, but not invariably, with a more or less pronounced decrease in the blood phosphates. Our findings tend to support the suggestion of Eadie, Macleod, and Noble (29) that there is a definite renal threshold for phosphate.

*5. Effect upon Ketosis and Ketonuria of Glucose Given Alone and with Insulin.*

Ringer (2) was the first to report that insulin given with glucose abolishes the ketonuria of phlorhizin diabetes. Our results show that glucose alone will accomplish the same result, and to quite a comparable degree. Our findings in this respect cannot be explained by temporary retention of the ketone bodies in the blood or other tissues. Not only is there no compensatory after excretion, but when the acetone bodies have disappeared from the urine they can no longer be found in the blood.

This observation is of fundamental importance to the question of interdependability of fat and carbohydrate metabolism. The universally accepted view that glucose must be oxidized to enable complete and normal oxidation of the end-products of fatty acid metabolism is expressed in one of the maxims of physiology, *Fat is burned in the flame of carbohydrate.* The whole of the important work of Shaffer (30) and his associates upon the ketolytic reaction involves this conception. Greenwald, Gross, and Samet (31) have only recently restated this view: "As is well known, acetoacetic acid is not readily, if at all, oxidized unless glucose is also

being oxidized. For the latter process and, consequently, for the former, the presence of insulin is necessary." If our results are confirmed—and it would appear that they are of themselves reasonably exhaustive and convincing—the present conception of keto-antiketogenic relationships must be revised or extended.

Quantitative comparison of the effects of glucose alone and with insulin in our experiments is difficult, since the severity of the ketosis in phlorhizin diabetes is progressively greater as the experiment continues, and the degree of effect produced thereupon by either glucose alone or with insulin is apparently determined by the relative concentrations of both factors in the reaction. Thus, Dog 17, which on the 5th phlorhizin day excreted more than 10 gm. of ketone bodies expressed as acetone, had so severe a ketosis that even 60 gm. of glucose given with 45 units of insulin failed to completely abolish the ketonuria. Yet on the preceding day a single dose of 30 gm. of glucose alone had reduced the hourly excretion of total acetone from 330 to 32 mg. Dog 21, which eliminated in the urine of the 24 hour period ending at 9.30 a.m., May 23, 5.159 gm. of total acetone, was given at 9.35 a.m. a solution of 30 gm. of glucose, and this dose was repeated 3 hours later; the urine from 1.30 p.m. to 4.00 p.m. (at which time the animal was killed) was negative for ketone bodies. On May 21, when the same quantities of glucose were given to this dog, a ketonuria of 139 mg. of total acetone per hour was completely abolished for 10 hours. It is of interest to note that in Dog 13, where, starting from a level of 50 mg. per hour a single dose of 30 gm. of glucose had in 2 hours rendered the urine ketone-free, 60 gm. of glucose and 40 units of insulin had not, in 6 hours, entirely abolished the ketonuria of the following day from an initial level of 106 mg. per hour.

Lusk (19) has called attention to the usual mild ketosis in phlorhizin diabetes, and has suggested that the intensity of ketosis depends upon the species of the reacting organism. Some phlorhizinized dogs, however, are susceptible to a very severe ketosis, as indicated in our results. When the relative weights of the animals compared are taken into account the extent of the ketonuria in Dogs 8, 9, 21, and particularly Dog 17, is of the same order as that occurring in the more severe cases of human diabetes.

*6. Effect upon Physical Condition of the Animals of Glucose Given Alone and with Insulin.*

We have observed repeatedly that when glucose is given to phlorhizinized dogs there is a temporary, but quite pronounced, improvement in the general condition of the animals. Dogs so weak that they are unable to get upon their feet or stand unaided, and with marked muscle incoordination, after glucose ingestion recover for a time practically a normal activity and show a normal interest in their surroundings. Several unusually impressive demonstrations of this effect occurred in the present experiments. Dog 21, for example, on the 4th day of phlorhizin and the 7th of fasting, was so prostrated that it remained without moving when placed in any position in the cage. Doubt was felt that the animal would last out the experiment. The blood pressure was so low that we were unable to take blood from the jugular vein. Glucose was then given in two 30 gm. doses. Within 2 hours of the first glucose treatment the dog was definitely much stronger; got up and walked about the cage; wagged tail; took water. The blood pressure was higher, so that blood was now readily taken from the vein. Throughout the day the dog's condition remained very good. Next morning, however, the dog was again in a critical state. During the course of the morning the dog had several short fits of howling, with eyes wild and distended. During one such seizure the urine was incontinent. The dog seemed temporarily insane. Later, in the afternoon, a critical state of air hunger developed. The dog panted for breath with mouth open and tongue out. The respiration was too rapid to be counted accurately (above 125 per minute). The dog was left during the night with no expectation that it would survive. The following morning, however, the respiration rate had diminished very considerably although the dog was still so weak that it could not stand. When supported the animal took water eagerly. Glucose and insulin were now given in divided doses, as shown in the protocol, and the animal again very promptly recovered. Within 2 hours it was able to support itself and move about the cage, take water unaided, and for the first time in 24 hours responded to call.

It is our distinct impression that while glucose alone invariably improves the physical state of the phlorhizinized dog for a short

period, the subsequent condition of the dog is worse than if no glucose had been given. We are now of the opinion that the temporary improvement coincides with, and is due to, temporary abolition of the ketosis and diminution of the acidosis. The subsequent unfavorable results may possibly be accounted for as follows: The ketosis in phlorhizin diabetes develops gradually, and the animal is able to some extent to accommodate its physiology to the slow progressive changes. After the effect of ingested glucose has worn off, however, the ketosis picks up promptly at about the level at which it was suddenly abolished. Apparently the dog is not as well able to tolerate a sudden severe ketosis as a gradually developing one.

When insulin is given with glucose the first effect is also a definite improvement in the physical state of the animal. But as the blood sugar falls under the influence of insulin to a level of about 50 mg. per cent hypoglycemic reactions supervene. The change is usually remarkably sudden, and is preceded by a short period in which the animal is notably more alert and active and may show some degree of excitability. Dog 13 (Table II), for example, had shown the usual improvement following glucose and insulin administration. At 5.30 p.m. urine was taken by catheter, and when the animal was returned to the cage it appeared to be in excellent condition. 10 minutes later the dog was observed stretched out in the cage, apparently unconscious, and had defecated in the lying position.

In many instances the first indication we have observed of the hypoglycemic syndrome is an increased respiration and heart rate.

*7. Glycogen Formation after Giving Glucose Alone and with Insulin.*

It was suggested both by Ringer (2) and in our first paper that insulin leads to glycogen storage in the phlorhizinized dog. While we were studying this point Cori (32) reported experiments in which he demonstrated glycogen formation in fasting phlorhizinized rabbits and cats under the influence of insulin. Our present results extend this observation to phlorhizinized dogs, and confirm the glycogenic action of insulin in these animals.

A question of greater interest in our work was the comparable effect upon glycogen formation of glucose given alone to phlor-

hizinized dogs. The inference has been very general that phlorhizinized animals do not have the capacity to store glycogen.[4] All phlorhizin experiments are prefaced by a fore period for the exhaustion of the glycogen reserves; and various procedures, such as inducing convulsions or shivering, anesthesia, violent exercise, or injection of adrenalin (Sansum and Woodyatt (38)), have been employed to completely deglycogenize phlorhizinized animals preliminary to feeding tests whose interpretation rests upon the assumption that none of the carbohydrate formed was retained.[5]

The effects of glucose given alone and with insulin upon glycogen

[4] Direct experiments have seemed to justify this assumption. Csonka (33) found no increase in the glycogen content of muscle or liver of phlorhizinized dogs killed within 2½ to 4 hours after feeding 50 gm. of glucose. Woodyatt (34) observed only a trace of glycogen in the liver, and 0.22 per cent in the muscle, of a phlorhizinized dog following 12.9 gm. of glucose given intravenously. Palmer (18) reported two dogs which were made glycogen-free by phlorhizin and adrenalin administration. Each dog then received 4 gm. of glucose per kilo of body weight. Following this procedure, only a trace of glycogen was found in muscle, while the liver was negative, in one dog; in the other dog the liver contained 0.16 per cent, and the muscle 0.34 per cent of glycogen. Epstein and Baehr (35), however, have claimed that phlorhizin stimulates the liver to build up glycogen, and when the glycosuric factor is eliminated by removal of the kidneys the effect of phlorhizin upon the liver becomes manifest in a rapid synthesis of glycogen, even in a state of extreme fasting. Torök (36) found that phlorhizinized dogs maintained in nitrogen equilibrium on diets of ox flesh and potatoes, and killed 6 days after phlorhizin, showed unusually high glycogen content of liver and muscles. Ringer, Dubin, and Frankel (37) have offered evidence that fasting phlorhizinized dogs, even after they have been completely deglycogenized, possess the power of glycogen formation. Cori (32), also, has recently speculated upon the possibility that sugar given to phlorhizin-poisoned animals may be temporarily stored as glycogen, even though the sugar is finally quantitatively recovered in the urine. We have observed in many experiments on phlorhizinized dogs that the glucose-nitrogen ratios for short periods show considerable fluctuation, usually being lower at night and higher in the early day periods when the animal is more active. We have also noted that after periods of forced exercise, or other procedure calculated to deglycogenize the animal, the glucose-nitrogen ratios are for a time lower than the average.

[5] In view of the fact now established that phlorhizinized dogs are capable of extensive glycogen synthesis and storage, it would seem that procedures calculated to deglycogenize completely such animals are contraindicated as a preliminary to subsequent feeding tests, where the recovery of "extra" sugar is the critical factor.

formation in the animals of our experiments are compared in Table XIII. It will be observed that we found very marked increases in the glycogen content of muscles and liver not only when glucose and insulin were given together, but when glucose alone was given. Our values for muscle glycogen may be compared with those obtained by Ringer, Dubin, and Frankel (37) on

TABLE XIII.

*Glycogen Formation in Phlorhizinized Dogs Following the Administration of Glucose Alone and with Insulin.*

Glucose given in two equal doses at an interval of 3 hrs.; insulin given in four doses at 90 minute intervals, beginning at the time of the first glucose dosage.

| | Dog 12. | Dog 13. | Dog 14. | Dog 16. | Dog 18.* | Dog 20. | Dog 21. |
|---|---|---|---|---|---|---|---|
| Interval between last dose of glucose and death of animal, *hrs.* | 4¼ | 3½ | 4 | 3 | 4 | 3½ | 3½ |
| Weight at death, *kg.* | | 11.6 | 10.5 | 12.0 | 10.9 | 13.3 | 12.3 |
| Weight of liver, *gm.* | | 435 | 404 | 430 | 374 | 442 | 450 |
| Blood sugar at death, *mg. per 100 cc.* | 76 | 50 | 50 | 233 | 111 | 179 | 234 |
| Glucose given, *gm.* | 50 | 60 | 60 | 60 | 60† | 60 | 60 |
| Insulin given, *units.* | 40 | 40 | 40 | None. | None. | None. | None. |
| Muscle glycogen, *per cent.* | 0.540 | 0.705 | 0.560 | 0.467 | 0.413 | 0.870 | 0.497 |
| Liver glycogen, *per cent.* | 3.35 | 3.39 | 2.88 | 1.96 | 1.23 | 3.65 | 1.11 |
| "Extra" glucose in urine for the period, *gm.* | 7.13 | 13.93 | 22.50 | 25.37 | 25.0 | 30.0 | 33.6 |

* Protocol not shown.

† Dog vomited 1½ hrs. before death, 22.46 gm. of the glucose given being thus recovered.

the muscle of fasting phlorhizinized dogs. These workers found the average muscle glycogen of twelve phlorhizinized dogs after 4 days of diabetes to be 0.228 per cent, the maximum value being 0.319 per cent. For seven dogs, after 5 days of phlorhizin diabetes, the average value of the muscle glycogen was 0.155 per cent, with a maximum in one dog of 0.315 per cent. (Our dogs

were killed at the end of the 4th or 5th day of diabetes.) The liver glycogen of fasting phlorhizinized dogs is practically nil. In this connection we may refer, also, to the recent results of Fisher and Lackey (39) on the glycogen content of liver and muscle of normal and depancreatized dogs. These workers found in normal, fed dogs a maximum liver glycogen of 2.73 per cent; minimum, 0.77 per cent. In two normal dogs fasted for 5 days, the liver glycogen was 0.43 per cent and 0.09 per cent, respectively. In depancreatized dogs fasted for 4 days, the liver glycogen was less than 0.1 per cent.

The average glycogen content of liver and muscles is higher in those dogs which received insulin with glucose, yet the highest individual values obtained were with Dog 20, which received glucose alone.

It should be noted in relation to the glycogen results that Dogs 16 and 21 had received during earlier periods of the experiments corresponding doses of glucose of which 92 and 88 per cent, respectively, had been recovered. The result of an earlier experiment on Dog 13, in which 60 gm. of glucose was given with insulin and 83 per cent of the glucose was recovered, should also be compared with the glycogen formation found in the same animal in the later experiment under the same conditions.

The most significant difference between the two groups of results is that, with insulin, glycogen formation occurs with a very low blood sugar concentration, while with glucose alone glycogen synthesis is associated with a hyperglycemia. Dog 13, whose tissues contained the highest concentrations of glycogen found in the dogs of the insulin-glucose group, was killed while in a comatose condition after extreme hypoglycemic symptoms. It is certainly striking that the hypoglycemic reactions induced by insulin may occur when the muscles and liver contain such ample carbohydrate supplies. The fact seems to us to bear importantly upon the mechanism of insulin action, emphasizing the glycogenic influence of insulin upon the glycogen-glucose equilibrium.

Any attempt to reconcile the quantities of sugar retained and glycogen stored is attended by many obviously uncertain factors. Nevertheless, data have been included in Table XIII which permit a rough calculation. Thus, Dog 13 had excreted 14 gm. out of 60 gm. of glucose given; and in the half-hour period imme-

diately preceding death, when the blood sugar was 50 mg. per cent, the sugar excretion rate was less than 0.5 gm. per hour. The liver, weighing 435 gm., contained 3.39 per cent of glycogen, or a total equivalent to about 15 gm. of glucose. The weight of the dog at death was 11.6 kilos of which 4 kilos may have been weight of muscle tissue. If the glycogen content of the muscles was 0.5 per cent higher than normal, something more than 20 gm. of the glucose ingested could here be located. These several sums would account for between 80 and 90 per cent of the glucose ingested.

## SUMMARY.

The following results have been observed after giving glucose alone, or with insulin, to fasting phlorhizinized dogs.

1. A diminution in nitrogen output in the urine of from 30 to 65 per cent, associated with a lowered non-protein nitrogen of the blood.

2. Little if any effect upon the rate of creatinine excretion.

3. A large decrease in, or complete abolition of, the excretion of inorganic phosphate, attended usually by a lowered concentration of the blood inorganic phosphates.

4. A large decrease in, or complete abolition of, the ketosis and ketonuria.

5. A pronounced, temporary improvement in the physical condition of the animals, followed by periods of abnormal weakness and prostration.

6. Extensive synthesis and storage of glycogen in muscles and liver.

No consistent quantitative difference in these results has been noted when equivalent amounts of glucose are given alone, and with insulin.

The average recovery of glucose when given alone, in eleven experiments, was 91 per cent. In six experiments the average recovery of glucose given with insulin was 72.5 per cent. The fate of the larger amounts of glucose retained under the influence of insulin has not been solved in these experiments. However, glycogen storage has been demonstrated to an extent that would seem to support the possibility of fat synthesis.

Several possibilities other than an increased oxidation of glucose

have been suggested to account for the elevation of respiratory quotient in phlorhizinized dogs following the administration of glucose and insulin together. Until these possibilities are eliminated it does not seem justifiable to interpret increases in the respiratory quotient as proof of the oxidation of glucose by the phlorhizinized dog.

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# THE EFFECT OF TEMPERATURE ON THE ACID-BASE-PROTEIN EQUILIBRIUM AND ITS INFLUENCE ON THE $CO_2$ ABSORPTION CURVE OF WHOLE BLOOD, TRUE AND SEPARATED SERUM.

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The influence of change of temperature on the carbonic acid-base-protein equilibrium in blood or serum involves three factors: (1) Change in solubility of $CO_2$ in serum or blood. (2) Change in the equilibrium constants of the buffer systems in the blood. (3) Change in the equilibrium constant for the dissociation of water.

The fact that the solubility of $CO_2$ changes with temperature has naturally been fully appreciated. On the other hand, the physiological significance of the change in the dissociation constant of water is more or less obscure but has been discussed in a recent paper by Austin and Cullen (1925). These topics will not be considered here.

The relations of temperature to the equilibrium constants of the buffer systems of the blood were discussed by Stadie and Martin (1924) whose work was confined to whole blood. Further work from this laboratory by Cullen, Keeler, and Robinson (1925) together with that presented in this paper have enabled us to extend the equilibrium relations as affected by temperature so as to include true serum and separated serum as well as whole blood. In addition the relations developed lead to a theoretical expression for one of the factors in the C correction of Cullen used to convert [pH] of serum at 20°C. to pH at 38° which shows the limitations of the C correction as a constant.

*Equilibrium Relations of the Buffers of the Blood.*

The equilibrium constants of the buffer systems of serum may be written as follows:

$$\begin{aligned} pH &= pK'_{[H_2CO_3]} + \log \frac{[BHCO_3]}{[H_2CO_3]} \\ &= pK'_{[H_2PO_4^-]} + \log \frac{[B_2HPO_4]}{[BH_2PO_4]} \\ &= pI + \frac{[BPr]}{\beta[Pr]} \\ &= pK'_{(x)} + \log \frac{BX}{HX} \end{aligned} \tag{1}$$

The portions of the above expression relating to carbonates and phosphates are logarithmic forms of the mass law equation, the former being the familiar Henderson-Hasselbalch equation. The last portion of the expression is a similar expression for any additional hypothetical weakly dissociated acid that may occur in serum under pathological conditions. There is evidence against the existence of any such acid in normal serum in significant quantity. The expression

$$pH = pI + \frac{[BPr]}{\beta[Pr]} \tag{2}$$

is a rearrangement of Van Slyke's buffer equation

$$[BPr] = \beta[Pr]\,(pH - pI) \tag{3}$$

where [BPr] = milli-equivalents of base bound by protein.

$\beta$ = the buffer constant for the particular serum or blood; *i.e.*, the change in milli-equivalents of base bound by 1 unit of [Pr] for 1 unit $\Delta$pH.

[Pr] = the concentration of protein for the serum or blood. For serum protein the unit of [Pr] is 1 gm. For hemoglobin it is 1 millimol.

[pI] = an empirical constant that for a single protein is close to but not necessarily identical with the pH at the isoelectric point.

This equation (3) was first used by Van Slyke (1922) in his study of buffers and in his application of his general formula for buffers to the special case of hemoglobin and serum protein. It is used by Van Slyke, Wu, and McLean (1923) in their equations 54, 55, and 56. We rearrange it merely that it may be more readily compared with the other expressions in equations (1). This buffer equation of Van Slyke, applicable to any buffer system, happens to be especially convenient for the protein buffer of serum and whole blood over physiological pH range because of the fact observed by Van Slyke and his coworkers that over this range the base bound by protein [BPr] in serum or in reduced blood is approximately a linear function of pH and hence $\beta$[Pr] is a constant over this pH range. The values for pI and $\beta$ are different for cells and for serum and hence for whole blood.

From the studies of Van Slyke, Hastings, and Neill (1922) and of Stadie and Martin (1924), it is clear that for oxygenated hemoglobin there is a deviation from the linear relation of [BPr] to pH. However, one can calculate from Fig. 2 of Stadie and Martin that between pH of 7.0 and 8.0 for a blood of normal hemoglobin content the deviation from a *mean* straight line drawn through the true values does not exceed $\pm$ 0.6 mM [BPr] at any point. For the purpose of this paper then the linear relation for fully oxygenated as well as reduced blood is assumed as an approximation.

The base in the blood is partly bound by strongly dissociated acids which at all physiological pH values and temperatures exist as salts and bind a constant amount of base. The remainder is distributed between the various weakly dissociated acids of equations (1). This may be expressed as follows:

$$[B] = [BA] + [BHCO_3] + [BPr] + ([BH_2PO_4] + [B_2HPO_4] + [BX]) \quad (4)$$

where A includes the anion of all strongly dissociated acids, chiefly Cl′, and where the part in parenthesis is negligibly small in normal blood and serum as compared with the $[BHCO_3]$ and [BPr]. Under these conditions base bound as $[BHCO_3]$ + [BPr] may be considered a constant which we will call [b] for a given sample of blood or separated serum regardless of changes within physiological limits of $[O_2]$, $[CO_2]$, pH, or t°. This is indicated diagramatically in Fig. 1 where the distribution of base

between Pr′ and $HCO_3'$, as it is equilibrated with varying tensions of $CO_2$ with consequently varying pH, is shown for the same separated serum at 20° and 38°.

Fig. 1 emphasizes the fact that the slope and location of the $CO_2$ absorption curve of a serum (and the same is true of whole

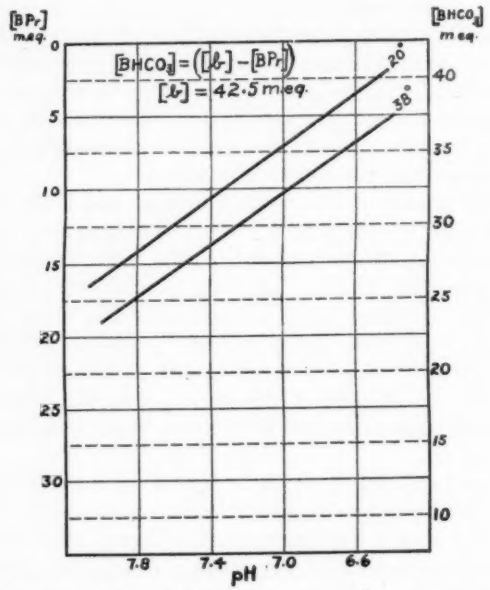


Fig. 1. Showing the relation of $CO_2$ absorption curve of a separated serum expressed as $[BHCO_3]$ against pH to the curve of [BPr] against pH. $[BHCO_3] + [BPr] = [b]$, a constant (approximately) for any given separated serum.

blood) is determined essentially by the constant value for $[BHCO_3]$ + [BPr] and by the value of [BPr] at any pH. The location and slope of the $CO_2$ absorption curve either of whole blood or of separated serum is essentially determined by the location and slope of the curve for [BPr]. In other words the carbon dioxide dissociation curve of separated serum is determined by the concen-

tration of total $CO_2$, the concentration of protein, and its constants pI and $\beta$. The relation, therefore,

$$\Delta[BHCO_3] = -\Delta[BPr] \tag{5}$$

under the limitations stated is necessarily true.

For true serum, subjected to changes in $p_{CO_2}$, pH, or temperature while still in contact with the cells, that is as a phase of whole blood, the relationship between $[BHCO_3]$ and [BPr] is less simple. In true serum under these conditions with changes in [BPr] of cells and serum there is migration of all anions including the quantitatively important anion Cl′ through the cell membrane in amounts determined as shown by Van Slyke, Wu, and McLean by the total amount of each anion in the blood and the requirements of the Donnan equilibrium. Under these conditions $[BA]_{TS}$ is not constant and $\Delta(BHCO_3)_{TS}$ does not equal $-\Delta[BPr]_{TS}$ If we neglect changes in the relative volume of cells and serum and changes in the Donnan ratio of anions in cells and serum which are known to be small over narrow pH ranges, the ratio of concentration of $[BHCO_3]_{TS}$ to $[BHCO_3]_C$ and therefore to $[BHCO_3]_B$ will be constant, and as an approximation we may write

$$\Delta[BHCO_3]_{TS} = \Delta[BHCO_3]_B = -\Delta[BPr]_B \tag{6}$$

This question will be discussed at greater length below. Therefore, while changes in $[BHCO_3]$ of separated serum are a measure of changes in [BPr] of separated serum, changes in $[BHCO_3]$ of true serum at 38°C. and of whole blood are both a measure of changes in [BPr] of whole blood. The quantitatively important change in [BPr] of whole blood is the change in base bound by hemoglobin. The slope and location of the $CO_2$ absorption curve is determined therefore by equations (3) and (4) when the factors in the parenthesis of (4) are quantitatively negligible so that equation (5) holds. Since

$$pH = pI + \frac{[BPr]}{\beta[Pr]} \tag{2}$$

and if

$$[BHCO_3] + [BPr] = [b] \tag{7}$$

where [b] is a constant for the particular separated serum or blood we can write

$$pH = pI + \frac{[b] - [BHCO_3]}{\beta[Pr]} \tag{8}$$

Equation (8) defines the location of the $CO_2$ absorption curve expressed as $[BHCO_3]$ against pH when the constants pI, [b] and $\beta[Pr]$ are known. Conversely the curve of $[BHCO_3]$ against pH determines $\beta[Pr]$; for

$$pH - pH' = pI + \frac{[b] - [BHCO_3]}{\beta[Pr]} - \left(pI + \frac{[b] - [BHCO_3]'}{\beta[Pr]}\right) \tag{9}$$

or

$$\beta[Pr] = \frac{[BHCO_3]' - [BHCO_3]}{pH - pH'} \tag{10}$$

Further, if $\beta[Pr]$ is constant at all temperatures, then it will be obvious from equation (2) that if we select points on the curves at two temperatures having the same value for $[BHCO_3]$ it will be true that:

$$pH_t - pH_{t'} = pI_t - pI_{t'} \tag{11}$$

and we can evaluate the change of pI with temperature.

The equation developed above may be used to evaluate experimental data relating temperature and $CO_2$ absorption curves and in the present paper we present additional data on whole blood, true serum, and separated serum of dog and sheep at 20°C. and 38°C.

*Methods.*

Blood was drawn from heart of the dog, or jugular vein of the sheep under oil or from the rat by instant decapitation. The blood was defibrinated by stirring under oil with a glass rod.

Equilibration of blood or serum was performed by the first or second saturation method of Austin, Cullen, Hastings, McLean, Peters, and Van Slyke (1922) and the technique there described for separation of true serum was employed.

Electrometric pH was done by the method and with the standardization described by Cullen, Keeler, and Robinson (1925).

Colorimetric pH at 20° was read by the technique of Cullen (1922) and at 38° by that of Hastings and Sendroy (1924) using for both the latters' bicolor standards. Colorimetric readings are expressed with bracketed figures, $[pH]_{20}$ indicating the color given at 20° by the phosphate buffer of corresponding pH at 20° and $[pH]_{38}$ indicating the color given at 38° by the phosphate buffer of corresponding pH at 38°. Unbracketed pH figures indicate gasometric or electrometric pH. $[CO_2]$ analyses were performed by Van Slyke's method. Gasometric pH was calculated by the Henderson-Hasselbalch equation from data on separated serum or true serum using Bohr's $\alpha$ (0.975 $\alpha$ water) and $pK'_{38} = 6.10$, $pK'_{20} = 6.19$ (Cullen, Keeler, and Robinson)

## DISCUSSION.

Our experimental data are shown in Table I. From these data $[BHCO_3]$ was plotted against pH. The resulting slopes are given in Table I in the columns headed $\frac{\Delta[BHCO_3]}{\Delta pH}$ for each true serum, whole blood, and separated serum.

### *Effect of Temperature on $\beta[Pr]$.*

The effect of temperature on the slope of the $CO_2$ absorption curve of whole blood, *i.e.* upon the value of $\beta[Pr]_B$, is not completely settled by the data of Table I. Two of the four experiments show a greater slope at 38°C. than at 20°C., one the reverse outside the limits of experimental error. One gives within experimental limits the same slope. Such evidence neither proves nor disproves the assumption of Stadie and Martin that $\beta[Pr]$ does not vary with temperature and furnishes no evidence for abandoning for the present the assumption. We tentatively assume, therefore, that at both 20° and 38°

$$\Delta[BHCO_3]_B = -\Delta[BPr]_B \qquad (12)$$

total available base $[b]_B$ of blood being constant.

It will be seen from Table I that both at 20°C. and 38°C. the slope $\frac{\Delta[BHCO_3]}{\Delta pH}$ is constantly steeper for true serum than for the corresponding blood. The ratio of the slopes is $1.13 \pm 0.04$ with

TABLE I.

| Source. | Date. | Equilibration. | | | Serum. | | | | Blood. | | $\frac{\Delta[BHCO_3]}{\Delta pH}$ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Analyzed. | Calculated. | | | | | | | |
| | | Material. | t°C. | $p_{CO_2}$ | $[CO_2]$ | $[H_2CO_3]$ | $pK_s'$ | Gasometric. | $[CO_2]$ | $[O_2]$ capacity. | Serum. | Blood ($\beta$[Pr]). | Ratio $\frac{\text{serum}}{\text{blood}}$. |
| | | | | mm. | mM | mM | | pH | mM. | vols. per cent | | | |
| Dog 26. | Apr. 16. | Blood. | 19.9 | 14.8 | 20.37 | 0.75 | 6.19 | 7.61 | 16.11 | 20.9 | 29.2 | 26.2 | 1.12 |
| | | " | 19.9 | 36.2 | 28.76 | 1.84 | 6.19 | 7.36 | 23.60 | 20.9 | | | |
| | | " | 38.0 | 39.5 | 21.12 | 1.25 | 6.10 | 7.30 | 16.56 | 20.9 | 27.4 | 23.8 | 1.15 |
| | | " | 38.0 | 76.8 | 27.51 | 2.44 | 6.10 | 7.11 | 22.10 | 20.9 | | | |
| Dog 33. | Apr. 22. | Blood. | 20.4 | 15.0 | 25.63 | 0.75 | 6.19 | 7.71 | 19.90 | 21.0 | 25.0 | 22.4 | 1.12 |
| | | " | 20.4 | 36.6 | 33.70 | 1.83 | 6.19 | 7.43 | 27.10 | 21.0 | | | |
| | | " | 38.0 | 40.0 | 25.95 | 1.27 | 6.10 | 7.39 | 20.10 | 21.0 | | 23.1 | |
| | | " | 38.0 | 77.1 | Lost. | 2.45 | 6.10 | (7.19) | 25.73 | 21.0 | | | |
| Dog 33. | June 4. | Blood. | 19.7 | 14.4 | 19.64 | 0.73 | 6.19 | 7.60 | 16.01 | 16.7 | 21.6 | 20.2 | 1 07 |
| | | " | 19.7 | 36.4 | 26.82 | 1.86 | 6.19 | 7.32 | 22.67 | 16.7 | | | |
| | | " | 38.0 | 39.6 | 19.93 | 1.26 | 6.10 | 7.27 | 16.24 | 16.7 | 23.0 | 22.3 | 1 03 |
| | | " | 38.0 | 76.3 | 25.47 | 2.42 | 6.10 | 7.08 | 21.51 | 16.7 | | | |
| Sheep. | May 20. | Blood. | 20.2 | 15.2 | 20.46 | 0.76 | 6.19 | 7.60 | 17.56 | 15.8 | 15.8 | 15.1 | 1 05 |
| | | " | 20.2 | 37.6 | 26.34 | 1.89 | 6.19 | 7.30 | 23.10 | 15.8 | | | |
| | | " | 38.0 | 40.2 | 19.96 | 1.28 | 6.10 | 7.26 | 17.16 | 15.8 | 20.8 | 17.7 | 1.18 |
| | | " | 38.0 | 78.2 | 25.32 | 2.48 | 6.10 | 7.06 | 21.78 | 15.8 | | | |
| Dog 27. | Jan. 14. | Separated serum. | 20.0 | 58.7 | 29.1 | 2.97 | 6.19 | 7.13 | | | $\beta$[Pr] 5.1 | | |
| | | " | 20.0 | 8.3 | 22.6 | 0.42 | 6.19 | 7.91 | | | | | |
| | | " | 38.0 | 84.4 | 27.0 | 2.68 | 6.10 | 7.06 | | | 5.4 | | |
| | | " | 38.0 | 23.6 | 22.4 | 0.75 | 6.10 | 7.56 | | | | | |

no striking difference in the dog between the slopes at the two temperatures. Equation (12) as an approximation holds for true serum but could be more accurately written

$$\Delta[BHCO_3]_{TS} = k\,\Delta[BHCO_3]_B = -k\,\Delta[BPr]_B \quad (13)$$

We may also write at constant pH but changing temperature

$$_{t-t'}\Delta[BHCO_3]_{TS} = k\,(_{t-t'}\Delta[BHCO_3]_B) = -k\,(_{t-t'}\Delta[BPr]_B) \quad (14)$$

At present we may put $k = 1.1$.

That the slope of the true serum curve is greater than that for whole blood is to be expected from the following considerations.

For whole blood, if v is the volume of the cells we have

$$(1 - v)\,[BHCO_3]_{TS} + v[BHCO_3]_C = [BHCO_3]_B \quad (15)$$

Assuming v constant a similar expression could be written for a higher $CO_2$ tension so that for a small increase we may write

$$(1 - v)\,\Delta[BHCO_3]_{TS} + v\Delta[BHCO_3]_C = \Delta[BHCO_3]_B \quad (16)$$

or

$$\Delta[BHCO_3]_{TS} = \Delta[BHCO_3]_B + v(\Delta[BHCO_3]_{TS} - \Delta[BHCO_3]_C) \quad (17)$$

If the concentrations of $BHCO_3$ be referred to the water content of cells and serum denoted by w and w′ respectively, the Donnan ratio can be written

$$\frac{[BHCO_3]_C}{[BHCO_3]_{TS}} \cdot \frac{w'}{w} = r \quad (18)$$

or for a small change at constant r, w and w′

$$\Delta[BHCO_3]_{TS} = \frac{w'}{wr}\,\Delta[BHCO_3]_C \quad (19)$$

The data on pp. 844 and 845 of the paper by Van Slyke, Wu, and McLean (1923) show for horse blood that $\frac{w'}{wr}$ is always greater than 1.0 between pH 7.0 and 7.7. It follows then that $(\Delta[BHCO_3]_{TS} - \Delta[BHCO_3]_C)$ is finite and positive and equation (17) becomes

$$\Delta[BHCO_3]_{TS} = \Delta[BHCO_3]_B + x[BHCO_3] \text{ where x is positive;} \quad (20)$$

or as an approximation we may write

$$\Delta[BHCO_3]_{TS} = k\ \Delta[BHCO_3]_B = -k\ \Delta[BPr]_B \qquad (21)$$

where k = 1.1 approximately.

It would, of course, be possible to pursue this matter further and elaborate a definite exact theoretical expression relating $[BHCO_3]_{TS}$ to $[BHCO_3]_B$ in terms of protein concentration, buffer slope, water content, pH, r, and total anion concentration of serum and cells respectively. This would involve merely a rearrangement of the equations derived by Van Slyke, Wu, and McLean (1923). However, the establishment of the validity of this relation would be nothing more than an experimental confirmation of their hypotheses. Theoretically this would be no advance and practically for each blood the relation would be different because of variation in the constants. Peters, Bulger, and Eisenman's empirical solution of this problem is eminently satisfactory for practically all purposes.

In twenty-one determinations of the $CO_2$ curve of whole blood and true plasma on human blood at 38°C., Peters, Bulger, and Eisenman (1924) found a greater slope for plasma in nineteen cases. The ratio of the slopes in these nineteen cases was 1.08 in close agreement with the value found here.

The approximate position of the $CO_2$ absorption curve of true serum relative to the whole blood curve may be located if the value of $[BHCO_3]_{TS}$ at one point is known by drawing a line through this point on a $[BHCO_3]$ — pH plot having a slope 1.1 of the slope of the whole blood. Lacking the one point on the true serum curve the location of this line is more hazardous but may be approximated by the method outlined by Peters, Bulger, and Eisenman (1924). Employing the principles outlined here the true serum curve located at any one temperature may be approximately located at other temperatures.

For separated serum the one experiment shows within the limits of experimental error the same slope at 20° and 38°C.; *i.e.*, $\beta[Pr]_{SS}$ = constant, so that we may write at any temperature

$$\Delta[BHCO_3]_{SS} = -\Delta[BPr]_{SS} \qquad (22)$$

total available base $[b]_{SS}$ of separated serum being constant.

From the data of Table I certain approximations as to the effect of temperature upon the acid-base equilibrium may be justifiably drawn. Assuming as an approximation a linear relationship of $[BHCO_3]$ against pH at both temperatures, we calculate from the data of Table I the changes with temperature of $[BHCO_3]$ at constant pH, at constant $[H_2CO_3]$, and at constant $p_{CO_2}$ and also the changes of pH at constant $[BHCO_3]$, $[H_2CO_3]$, and $p_{CO_2}$. These relations are tabulated in Table II.

It will be seen that a rather constant temperature effect is the ${}_{20°-38°}\Delta pH$ at constant $[BHCO_3]$. This we have pointed out above, if the slope of $[BHCO_3]$ against pH be the same at the two temperatures, is a measure of ${}_{20°-38°}\Delta pI$ of whole blood when the $\Delta pH$ measured is that of true serum and of ${}_{20°-38°}\Delta pI$ of separated serum when the $\Delta pH$ measured is that of separated serum. The data suggest that the ${}_{20°-38°}\Delta pI$ is for whole blood and separated serum approximately:

$$\frac{\Delta pI}{\Delta t°} = \frac{\Delta pH \quad [BHCO_3] \text{ constant}}{\Delta t°} = -0.017 \text{ to } -0.023 \qquad (23)$$

If it is desired to approximate the position of a $[BHCO_3]$ curve of a serum or blood against pH at some other temperature (t′) when the curve at t is known, it can be done conveniently by means of equation (23) and the assumption that the slope of the $[BHCO_3]$ curve against pH at t′ is the same as at t.

In order to aid in visualizing the nature of these changes nomograms (Figs. 2 and 3) have been prepared of the relations in the true serum of Dog 33, June 4, and in the separated serum of Dog 27, January 14. It will be seen that while the interval between the lines is much greater in separated serum due to its smaller $\beta[Pr]$ value and consequently flatter $CO_2$ absorption curve, the slope of the lines in physiological ranges is almost the same for true serum and for separated serum. These nomograms also bring out the fact, pointed out by Austin and Cullen (1925) that with change in temperature from 38° to 20° at constant $p_{CO_2}$ the pH of serum varies very little. They pointed out, however, that there is a marked associated change in pOH.

TABLE II.

| Source. | Date. | Material. | Temperature. | $[BHCO_3]$ | pH | ΔpH at constant $[BHCO_3]$. $-\frac{\Delta pH}{°C.}$ per °C. | ΔpH at constant $[BHCO_3]$. Average. | Δ$[BHCO_3]$ at constant pH. pH | Δ$[BHCO_3]$ at constant pH. $-\frac{\Delta[BHCO_3]}{°C.}$ per °C. | Δ$[BHCO_3]$ and ΔpH at constant $p_{CO_2}$. $p_{CO_2}$ | Δ$[BHCO_3]$ and ΔpH at constant $p_{CO_2}$. $-\frac{\Delta[BHCO_3]}{°C.}$ per °C. | Δ$[BHCO_3]$ and ΔpH at constant $p_{CO_2}$. $-\frac{\Delta pH}{°C.}$ per °C. | Δ$[BHCO_3]$ and ΔpH at constant $[H_2CO_3]$. $[H_2CO_3]_0$ | Δ$[BHCO_3]$ and ΔpH at constant $[H_2CO_3]$. $-\frac{\Delta[BHCO_3]}{°C.}$ per °C. | Δ$[BHCO_3]$ and ΔpH at constant $[H_2CO_3]$. $-\frac{\Delta pH}{°C.}$ per °C. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | °C. | mM | | | | | mM | mm. | mM | | mM | mM | |
| Dog 26. | Apr. 16. | Blood. | 19.9 | 15.5 | 7.61 | 0.0171 | | | | | | | | | |
| | | " | 38.0 | 15.5 | 7.30 | | | 7.32 | 0.45 | 38 | 0.41 | 0.0017 | 1.50 | 0.20 | 0.0099 |
| | | " | 19.9 | 21.0 | 7.40 | 0.0182 | | | | | | | | | |
| | | " | 38.0 | 21.0 | 7.07 | | 0.0173 | | | | | | | | |
| | | True serum. | 19.9 | 19.6 | 7.61 | 0.0166 | | | | | | | | | |
| | | " " | 38.0 | 19.6 | 7.31 | | | 7.32 | 0.48 | 38 | 0.38 | 0.0039 | 1.50 | 0.17 | 0.0099 |
| | | " " | 19.9 | 25.5 | 7.41 | 0.0171 | | | | | | | | | |
| | | " " | 38.0 | 25.5 | 7.10 | | | | | | | | | | |
| Dog 33. | Apr. 22. | Blood. | 20.4 | 14.0 | 7.72 | 0.0188 | | | | | | | | | |
| | | " | 38.0 | 14.0 | 7.39 | | | 7.39 | 0.43 | 39 | 0.46 | 0.0006 | 1.60 | 0.22 | 0.0091 |
| | | " | 20.4 | 24.0 | 7.50 | 0.0182 | 0.0186 | | | | | | | | |
| | | " | 38.0 | 24.0 | 7.18 | | | | | | | | | | |
| | | True serum. | 20.4 | 24.6 | 7.72 | 0.0188 | | | | | | | | | |
| | | " " | 38.0 | 24.6 | 7.39 | | | 7.39 | 0.47 | 40 | 0.50 | 0.0000 | 1.26 | 0.24 | 0.0108 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dog 33. | June 4. | Blood. | 19.7 | 15.2 | 7.61 | 0.0188 | | | | | | | | | |
| | | " | 38.0 | 15.2 | 7.30 | | | | | | | | | | |
| | | " | 19.7 | 21.0 | 7.40 | 0.0182 | | | | | | | | | |
| | | " | 38.0 | 21.0 | 7.07 | | | | | | | | | | |
| | | True serum. | 19.7 | 19.6 | 7.61 | 0.0166 | | | | | | | | | |
| | | " " | 38.0 | 19.6 | 7.31 | | | | | | | | | | |
| | | " " | 19.7 | 25.5 | 7.41 | 0.0171 | | | | | | | | | |
| | | " " | 38.0 | 25.5 | 7.10 | | | | | | | | | | |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dog 33. | June 4. | Blood. | 19.7 | 15.2 | 7.61 | 0.0186 | 0.0181 | 7.30 | 0.38 | 38 | 0.36 | 0.0005 | 1.58 | 0.17 | 0.0093 |
| | | " | 38.0 | 15.2 | 7.27 | | | | | | | | | | |
| | | " | 19.7 | 20.0 | 7.37 | 0.0175 | | | | | | | | | |
| | | " | 38.0 | 20.0 | 7.05 | | | | | | | | | | |
| | | True serum. | 19.7 | 18.9 | 7.60 | 0.0186 | | 7.30 | 0.40 | 38 | 0.38 | 0.0005 | 1.58 | 0.18 | 0.0088 |
| | | " " | 38.0 | 18.9 | 7.26 | | | | | | | | | | |
| | | " " | 19.7 | 23.5 | 7.38 | 0.0175 | | | | | | | | | |
| | | " " | 38.0 | 23.5 | 7.06 | | | | | | | | | | |
| Sheep. | May 20. | Blood. | 20.2 | 15.5 | 7.62 | 0.0219 | 0.0205 | 7.28 | 0.34 | 39 | 0.33 | 0.0011 | 1.59 | 0.19 | 0.0095 |
| | | " | 38.0 | 15.5 | 7.23 | | | | | | | | | | |
| | | " | 20.2 | 20.0 | 7.39 | 0.0197 | | | | | | | | | |
| | | " | 38.0 | 20.0 | 7.04 | | | | | | | | | | |
| | | True serum. | 20.2 | 19.5 | 7.61 | 0.0219 | | 7.28 | 0.31 | 39 | 0.38 | 0.0011 | 1.59 | 0.19 | 0.0095 |
| | | " " | 38.0 | 19.5 | 7.22 | | | | | | | | | | |
| | | " " | 20.2 | 23.5 | 7.36 | 0.0186 | | | | | | | | | |
| | | " " | 38.0 | 23.5 | 7.03 | | | | | | | | | | |
| Dog 27. | Jan. 14. | Separated serum. | 20.0 | 22.0 | 7.94 | 0.0233 | 0.0214 | 7.40 | 0.11 | 40 | 0.13 | +ΔpH 0.0031 | 1.50 | 0.08 | 0.0065 |
| | | " " | 38.0 | 22.0 | 7.52 | | | | | | | | | | |
| | | " " | 20.0 | 25.0 | 7.35 | 0.0194 | | | | | | | | | |
| | | " " | 38.0 | 25.0 | 7.00 | | | | | | | | | | |

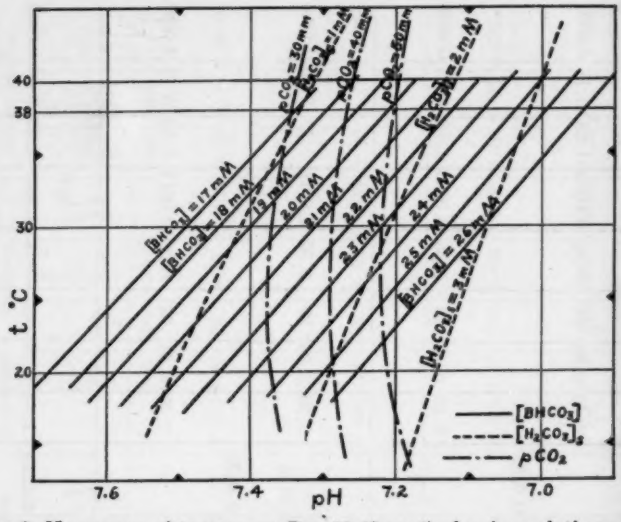


FIG. 2. Nomogram of true serum Dog 33 (June 4) showing relations of $[BHCO_3]_s$, $p_{CO_2}$, $[H_2CO_3]_s$, pH, and t°C.

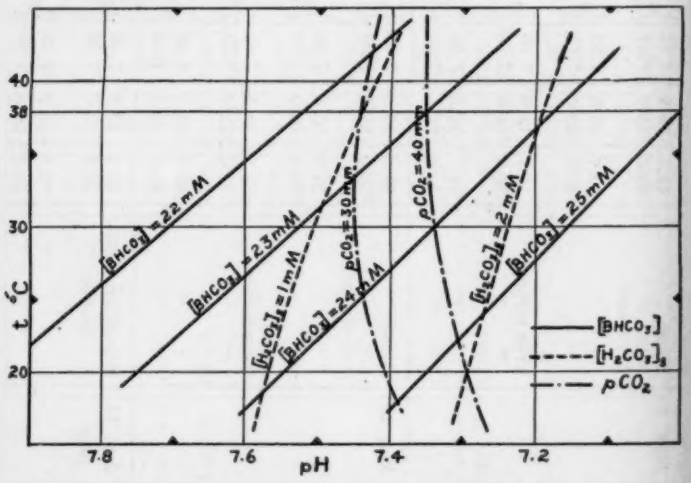


FIG. 3. Nomogram of separated serum Dog 27 (Jan. 14) showing relation of $[BHCO_3]_s$, $p_{CO_2}$, $[H_2CO_3]_s$, pH, and t°C.

*Change of pH of Serum with Temperature at Constant Total $CO_2$ and Constant Total Available Base: Calculation of $_t - _{t'}\Delta pH$.*

Separated serum equilibrated under known conditions at a given temperature, t°, or true serum separated from cells under equilibrium conditions and kept so that no loss or gain of $CO_2$ may occur (under oil or in mercury receivers) will on change of temperature assume a new equilibrium with consequent change of pH. The same thing is necessarily true for diluted serum. This change of pH enters into the C correction of Cullen's (1922) colorimetric method for pH of serum and plasma since the conditions defined are exactly those employed by Cullen in reading at 20°C. the [pH] of a serum equilibrated at 38°C. Austin, Stadie, and Robinson (1925) have shown elsewhere, however, that this pH change is not necessarily the most important cause of variation in the C correction. Disregarding this for the moment we will proceed to show the factors involved in the change of pH of serum with change of temperature from 38°C. to 20°C.; *i.e.*, $_t - _{t'}\Delta$pH and its possible variations. If we accept the evidence that with change in temperature the equilibrium of serum protein-carbonic acid systems is determined by change in pI of equation (3) and of pK′ and $\alpha$ of the Henderson-Hasselbalch equation, it is possible to calculate the $_t - _{t'}\Delta$pH under the above conditions as follows.

The amount of base in equivalents bound by serum protein at t° is given by the linear relation, equation (3)

$$[BPr] = \beta[Pr]\,(pH_t - pI_t) \tag{3}$$

where [Pr] = protein concentration gm. per liter
$\beta$ = protein buffer change in equivalents of base bound per gm. of protein per 1 unit ΔpH.
$pI_t$ = pH close to but not necessarily identical with the isoelectric point at t°C.

The equivalents of base bound by $HCO_3^-$ at t° are given by the familiar equation

$$[BHCO_3] = \frac{k_t'}{k_t' + [H^+]_t}\,[CO_2] \tag{24}$$

At equilibrium the sum of the base [BP] + [$BHCO_3$] is [b]

$$[b] = \beta[Pr]\,(pH_t - pI_t) + \frac{k_t'}{k_t' + [H^+]_t}\,[CO_2] \tag{25}$$

At t'°C. if no loss of $CO_2$ or change of protein concentration occurs

$$[b] = \beta[Pr](pH_{t'} - pI_{t'}) + \frac{k'_{t'}}{k'_{t'} + [H^+]_{t'}}[CO_2] \quad (26)$$

Equating, and assuming the total available base [BP] + [$BHCO_3$] which we call [b] unchanged, we have

$$({}_{t-t'}\Delta pH - {}_{t-t'}\Delta pI)\,\beta[Pr] = \left(\frac{k'_{t'}}{k'_{t'} + [H^+]_{t'}} - \frac{k'_t}{k'_t + [H^+]_t}\right)[CO_2] \quad (27)$$

Equation (27) shows that ${}_{t-t'}\Delta pH$ in serum (and likewise in diluted serum) at constant [$CO_2$] and [b] is dependent on the relation of the total [$CO_2$] to $\beta$[Pr], the magnitude of $\Delta pI$ with temperature, the change in k' (the dissociation of carbonic acid) with temperature and also upon the initial value of [$H^+$] or pH. The relation of $k'_t$ and $k'_{t'}$ of equation (27) to the $\frac{\Delta pK'}{\Delta t°}$ for the Henderson-Hasselbalch equation is as follows:

$$\log \frac{k'_t}{k'_{t'}} = pK'_{t'} - pK'_t = 0.09 \quad (28)$$

between 38°C. and 20°C.

Evidence that $\beta$ for hemoglobin is independent of temperature was presented by Stadie and Martin and as already pointed out our data furnish no evidence for change of $\beta$[Pr] of serum protein or whole blood with temperature. A graphical solution of equation (27) yields the graphs shown in Fig. 4, where abscissæ are initial $pH_{38}$, ordinates are ${}_{t-t'}\Delta pH = pH_{20} - pH_{38}$. It is at once evident that ${}_{t-t'}\Delta pH$ is not a constant. Variations in initial pH from 7.0 to 8.0 cause variations as large as 0.16 in ${}_{t-t'}\Delta pH$. Changes in protein buffer of serum well within the limits of variations observed in different species cause considerable change in the values of ${}_{t-t'}\Delta pH$ as well as in the importance of change in initial pH. Thus for a serum with [$CO_2$] = 20 mM, initial pH = 7.4 and $\beta$[Pr] = 6, we find ${}_{20-38}\Delta pH = 0.34$. A change in total [$CO_2$] of 10 mM causes change in ${}_{20-38}\Delta pH$ (at same initial pH) of 0.04 to 0.05 pH.

It should be pointed out that here over narrow physiological

ranges, 7.30 to 7.50, the changes in $_{20}-_{38}\Delta pH$ are small (0.04 to 0.05 pH). However, the use of a constant value for Cullen's C correction (which involves $_{20}-_{38}\Delta pH$) in experiments on separated serum saturated at different $CO_2$ tensions is associated with greater variations of $_{t}-_{t'}\Delta pH$ because lower initial pH is associated with higher $[CO_2]$. From Fig. 4 it will be seen that lowering initial $pH_{38}$ and increasing $[CO_2]$ both tend to diminish $_{20}-_{38}\Delta pH$; we have here, therefore, a summation of effects. For true serum the $\Delta [CO_2]$ at different initial $pH_t$ values is still greater with greater alteration in $_{20}-_{38}\Delta pH$ at different initial pH values.

The use of a constant Cullen's C correction therefore within physiological ranges introduces the possibility of errors due to

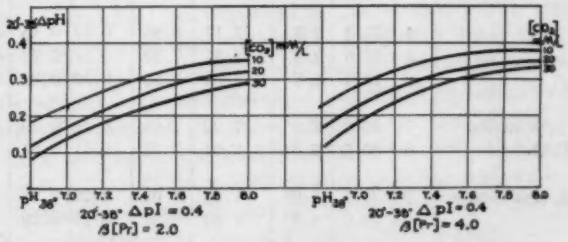


FIG. 4. Graphs showing $_{20-38}\Delta pH$ when separated serum undergoes change of temperature from 38° to 20°C. at constant total $[CO_2]_b$ from various initial $pH_{38}$ values. Graphs are drawn for three different $[CO_2]_b$ values and for two values for $\beta[Pr]$.

variation in $_{t}-_{t'}\Delta pH$ of from 0.06 to 0.09 pH units between 20° and 38°C. In experimental work on blood or serum over wider pH ranges still larger errors would be introduced as pointed out above.

Equation (27) must be valid, as already pointed out, if equation (3) is valid and if change in temperature affects only the constants pK′ and $\alpha$ of the Henderson-Hasselbalch equation and pI while $\beta[Pr]$ remains constant independent of temperature.

However, since the special interest of this aspect of the paper lies in the $_{20}-_{38}\Delta pH$ of serum under conditions of constant total $[CO_2]$ and [b], experiments were done to test equation (27) under these special conditions. The data are given in Table III.

The experiments are on both separated and true serum and

divide themselves into two groups, Nos. 1 to 12 and Nos. 13 to 19. The sample of serum was equilibrated at 38° and its gasometric $pH_{38}$ calculated from the Henderson-Hasselbalch equation.

TABLE III.

*Effect of Temperature on pH of Serum at Constant $[CO_2]$.*

| Source. | Experiment No. | $[CO_2]$ | $\beta[Pr]$ | pH | | $_{20-38}\Delta pH$ | | Difference observed—calculated. |
|---|---|---|---|---|---|---|---|---|
| | | | | 20° | 38° | Observed. | Calculated. | |
| | | mM | mM | | | | | |
| Dog 27. | 1 | 27.0 | 4.0 | 7.32 | 7.08 | 0.24 | 0.22 | +0.02 |
| | 2 | 24.5 | 4.0 | 7.65 | 7.40 | 0.25 | 0.29 | −0.05 |
| | 3 | 22.4 | 4.0 | 7.80 | 7.58 | 0.22 | 0.33 | −0.11 |
| Sheep 1. | 4 | 23.2 | 2.3 | 7.17 | 6.98 | 0.19 | 0.15 | +0.04 |
| | 5 | 21.6 | 2.3 | 7.57 | 7.30 | 0.27 | 0.23 | +0.04 |
| | 6 | 19.4 | 2.3 | 7.90 | 7.60 | 0.30 | 0.32 | +0.02 |
| Same with added $NaHCO_3$ | 7 | 46.2 | 2.3 | 7.22 | 7.08 | 0.14 | 0.10 | +0.04 |
| | 8 | 40.6 | 2.3 | 7.94 | 7.73 | 0.21 | 0.21 | 0.00 |
| Dog 33, true serum. | 9 | 25.5 | 4.0* | 7.28 | 7.08 | 0.20 | 0.22 | −0.02 |
| | 10 | 19.9 | 4.0* | 7.54 | 7.27 | 0.27 | 0.29 | −0.02 |
| Sheep 1, true serum. | 11 | 25.3 | 2.3* | 7.30 | 7.06 | 0.24 | 0.16 | +0.08 |
| | 12 | 19.9 | 2.3* | 7.57 | 7.26 | 0.31 | 0.24 | +0.07 |
| | | | | [pH] | [pH] | | | |
| Sheep 1. | 13 | 31 | | [7.22] | [7.10] | 0.12 | | |
| | 14 | 24 | | > [7.95] | [7.75] | >0.20 | | |
| Rat pooled serum. | 15 | 26 | | [7.37] | [7.21] | 0.16 | | |
| | 16 | 24 | | [7.67] | [7.47] | 0.20 | | |
| Dog 27. | 17 | 25 | | [7.22] | [7.05] | 0.17 | | |
| | 18 | 19 | | [7.61] | [7.40] | 0.21 | | |
| | 19 | 17 | | [7.87] | [7.63] | 0.24 | | |

* Assumed.

The color reading $[pH]_{20}$ at 20°C. of each sample was determined colorimetrically. The gasometric $pH_{20}$ and the correction $([pH]_{20} - pH_{20})$ were determined independently on samples of the same serum by equilibration at 20°C. over a range of pH. These

($[pH]_{20} - pH_{20}$) corrections were used to correct the other 20° colorimetric readings on the same serum equilibrated at 38° so that the values given as $pH_{20}$ in Table III are essentially gasometric $pH_{20}$ values. $\beta[Pr]$ was determined from the slope of the serum $CO_2$ absorption curves. The "calculated ${}_{20} - {}_{38}\Delta pH$" values are obtained from Fig. 4.

In the second group of observations (experiments 13 to 19) no ($[pH]_{20} - pH_{20}$) corrections were determined so that only the colorimetric readings of the diluted serum at 20° and 38°C. are given. Direct comparison therefore cannot be made with the calculated ${}_{20} - {}_{38}\Delta pH$ (see Austin, Stadie, and Robinson, 1925). The experimental data with the exception of No. 3 are in complete qualitative as well as quantitative agreement with the calculated values. ${}_{t} - {}_{t'}\Delta pH$ as observed is not constant but regularly (except No. 3) increases with pH. Increasing total $CO_2$ (Nos. 4 to 8) decreases ${}_{20} - {}_{38}\Delta pH$ roughly about 0.04 per 10 mM increase of $CO_2$ per liter. The calculated ${}_{t} - {}_{t'}\Delta pH$ is in agreement with the observed for most of the observations. In Nos. 11 and 12 the difference is outside the experimental error but calculated and observed ${}_{t} - {}_{t'}\Delta pH$ vary with change in $[CO_2]$ and with initial $pH_{38}$ in the same direction and to the same extent. No reasons for the discrepancies in Nos. 3, 11, and 12 are known.

## SUMMARY.

1. The dependence of the $CO_2$ absorption curve of serum or blood upon the base bound by protein at varying pH is discussed and the relation stated in equations (8) and (10).

2. The change in $CO_2$ absorption curve between 38° and 20°C. is determined experimentally for whole blood, true serum, and separated serum in dog and sheep.

3. Our data furnish no evidence that the value of $\beta[Pr]$ is altered consistently by this change of temperature.

4. On the assumption that $\beta[Pr]$ is the same at both temperatures one can evaluate the change of pI of equations (3) or (8) with temperature.

5. It is found that approximately for whole blood, true serum, and separated serum

$$\frac{\Delta\ pI}{\Delta\ t^\circ} = \frac{\Delta\ pH\ _{[BHCO_3]\ constant}}{\Delta\ t^\circ} = -0.017 \text{ to } -0.023.$$

6. When it is desired to approximate the position of a $[BHCO_3]$ curve against pH for either separated serum, true serum, or whole blood at another temperature, t′, when the curve at t is known, it can best be done by means of the equation in conclusion 5 and the assumption that the slope of the $[BHCO_3]$ curve against pH at t′ is the same as at t.

7. The slope of the true serum curve is found to be higher than that of the corresponding blood both at 20°C. and 38°C. The reason for this is discussed and an empirical relation

$$\Delta [BHCO_3]_{TS} = 1.1 \Delta [BHCO_3]_B$$

given relating the two slopes at any temperature.

8. The change in pH of a separated serum or whole blood undergoing change in temperature at constant $[CO_2]$ is calculated and the influence of initial pH, initial $[CO_2]$, and initial value for $\beta[Pr]$ is shown.

9. This change in pH (which enters into the C correction of Cullen's colorimetric method) may vary 0.09 pH units within physiological ranges with change in temperature from 20° to 38°, and is a factor therefore in the variation of the C correction.

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